



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : <b>C12N 9/00, 15/52, A61K 37/395 C07K 7/06, 15/28</b>		A1	(11) International Publication Number: <b>WO 93/03146</b> (43) International Publication Date: <b>18 February 1993 (18.02.93)</b>
(21) International Application Number: <b>PCT/US92/06283</b> (22) International Filing Date: <b>30 July 1992 (30.07.92)</b>		(74) Agents: WIXON, Henry, N.; Hale and Dorr, 1455 Pennsylvania Avenue, N.W., Washington, DC 20004 (US) et al.	
(30) Priority data: <b>738,040 30 July 1991 (30.07.91)</b>		US	(81) Designated States: AU, CA, FI, JP, KR, NO, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).
(60) Parent Application or Grant (63) Related by Continuation <b>US 738,040 (CIP) Filed on 30 July 1991 (30.07.91)</b>		Published <i>With international search report.</i>	
(71)(72) Applicant and Inventor: RAPORT, Basil [US/US]; 1 Lower Via Casitas, Greenbrae, CA 94904 (US).			
(54) Title: DISEASE ASSOCIATED B-CELL EPITOPE OF TPO AND USE THEREOF			
(57) Abstract <p>Disease associated B-cell epitopes of human thyroid peroxidase are described, particularly, a nine amino acid sequence corresponding to amino acids 713-721 of recombinant human thyroid peroxidase.</p> <p><i>Exha copy f. 102 103 104</i></p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

5

DISEASE ASSOCIATED B-CELL EPITOPES OF  
TPO AND USE THEREOF

10

15

Background of the Invention

20

Part of the work leading to this invention was made with U.S. Government funds. The U.S. Government has certain rights in this invention.

25

Field of the Invention

30

The present invention relates to the field of molecular biology and immunology. More particularly, the invention relates to the production of recombinant human thyroid peroxidase in non-thyroidal eukaryotic cells. The invention is further related to methods of using recombinant human thyroid peroxidase, and, in particular, to methods of using recombinant human thyroid peroxidase in diagnosis of immune disorders such as Hashimoto's thyroiditis.

35

Brief Description of the Related Art

Hashimoto's thyroiditis is the most common autoimmune endocrinopathy, affecting, at least subclinically, up to 15% of the adult female population (Volpe, R., In Werner's The Thyroid, 5th Edition (Ingbar, S.H., et al., Eds.), J.B. Lippincott Co., Philadelphia, pp. 1266-1291 (1986); Gordin, A., et al., Acta Endocrinol. 90:33-42 (1979)). Antibodies against a number of thyroid antigens are present in the sera of these patients, including thyroglobulin and the thyroid "microsomal" antigen (Doniach, D., et al., Clin. Endocrinol. Metab. 8:63-80 (1979); Weetman, A.P., et al., Endocr. Rev. 5:309-355 (1984)). Other antigens of lesser, or uncertain, importance, include the second colloid antigen (Doniach, D., et al., Clin. Endocrinol. Metab. 8:63-80 (1979)), tubulin (Rousset, B., et al., Clin. Exp. Immunol. 52:325-332 (1983)), DNA (Katakura, M., et al., J.Clin. Endocrinol. Metab. 64:405-408 (1987)) and Autoimmune Thyroid Disease-Related Antigen I (ATRA I) (Hirayu, H., et al., J. Clin. Endocrinol. Metab. 64:578-584 (1987)).

Antibodies against the microsomal antigen, which is expressed on the cell surface (Khoury, E.L., et al., Exp. Immunol. 45:315-328 (1981); Nilsson, M., et al., Molec. Cell. Endocrinol. 53:177-185 (1987)), are believed to be of greater importance than those against thyroglobulin in the pathogenesis of Hashimoto's thyroiditis. This is because antimicrosomal antibodies (MSA) are more closely associated with the active phase of the disease (Volpe, R., In Werner's The Thyroid, 5th Edition (Ingbar, S.H., et al., Eds.), J.B. Lippincott Co., Philadelphia, pp. 1266-1291 (1986); Bogner, U., J. Clin. Endocrinol. Metab. 59:734-738 (1984); Jansson, R., et al., Clin. Exp. Immunol. 63:80-86 (1986)) and are complement-fixing (Khoury, E.L., et al., Exp. Immunol. 45:315-

328 (1981)). These antibodies are, therefore, likely to initiate thyroid cellular damage.

A major recent discovery regarding Hashimoto's thyroiditis is that the previously ill-defined microsomal antigen is, at least in part, thyroid peroxidase (TPO), the primary enzyme involved in thyroid hormone synthesis. This conclusion was based on immunologic evidence (Czarnocka, B., FEBS Letters 109:147-152 (1985); Portmann, L., et al., J. Clin. Endocrinol. Metab. 61:1001-1003 (1985); Mariotti, S., et al., J. Clin. Endocrinol. Metab. 65:987-993 (1987)) and subsequently confirmed by the molecular cloning of the cDNA for these proteins (Magnusson, R.P., et al., J. Biol. Chem. 262:13885-13888 (1987); Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861 (1987); Libert, R., et al., EMBO J. 6:4193-4176 (1987); Kimura, S., et al., Proc. Natl. Acad. Sci. USA 84:5555-5559 (1987)) and the discovery that their derived amino acid sequences are the same (Libert, R., et al., EMBO J. 6:4193-4176 (1987); Seto, P., et al., J. Clin. Invest. 80:1205-1208 (1987)).

Prior to the present invention, a suitable preparation of recombinant TPO has not been available for studies on the presumed abnormalities in immune regulation in Hashimoto's thyroiditis, or for the demonstration of the specific B-cell and T-cell epitopes involved in this disease. In this respect, understanding of the molecular mechanisms involved in the pathogenesis of Hashimoto's thyroiditis lags far behind that of other immune disorders, such as myasthenia gravis, a disease for which pure antigen (the acetylcholine receptor) has been obtained and epitopes already defined (Tzartos, S.J., et al., Proc. Natl. Acad. Sci. USA 85:2899-2903 (1988); Hohlfeld, R., et al., J. Clin. Invest. 81:657-660 (1988)).

Human TPO (hTPO) immunopurified by monoclonal antibodies (mAbs) has been available, but is of limited value because of: (a) inadequate supplies of human thyroid tissue; (b) the

difficulties in purification of this membrane-bound antigen; and (c) contamination with other thyroid autoantigens such as thyroglobulin, which is highly abundant.

5       Fragments of hTPO have been generated as recombinant bacterial ( $\beta$ -galactosidase) fusion proteins, and reactivity of a number of Hashimoto patient sera with small fragments of TPO expressed as fusion proteins has been reported (Libert, R., *et al.*, EMBO J. **6**:4193-4176 (1987)). Those data, however, are difficult to interpret, because the plaque assays used require 10 extensive pre-adsorption of polyclonal antisera (Hirayu, H., *et al.*, J. Clin. Endocrinol. Metab. **64**:578-584 (1987)) and can yield false positive results.

15       For example, a reported fusion protein originally described as reactive with 19 of 20 Hashimoto patient sera (Libert, R., *et al.*, EMBO J. **6**:4193-4176 (1987), clone C2) has, upon immunopurification with anti- $\beta$ -galactosidase mAbs, been found to react with fewer Hashimoto patient sera in an ELISA assay (Dinsart, C., *et al.*, 17th Annual Meeting of the European Thyroid Association, Abstract #235 (1988)).

20       Thus, bacterial fusion proteins, too, have been of limited value because: (a) no combination of fragments has been found that reacts with all Hashimoto's sera; (b) the conformation of the fusion protein may differ from that of the native protein; and (c) the bacterial products may be toxic 25 when added to immune cells in culture.

#### SUMMARY OF THE INVENTION

30       In order to obtain full-length hTPO free of other potential thyroid antigens, the present inventor achieved expression of recombinant hTPO in non-thyroidal eukaryotic cells. Like native hTPO, this recombinant hTPO is enzymatically active, is expressed on the cell surface, and is not a fusion protein.

The recombinant hTPO of this invention is recognized in a specific manner by sera from patients with Hashimoto's thyroiditis that contain "antimicrosomal" antibodies. All 36 Hashimoto patient sera selected to represent a range of 5 antimicrosomal antibody levels seen in this disease were reactive with the eukaryotic-expressed recombinant hTPO of the invention.

It is an object of the present invention, then, to provide for a convenient and economical source of recombinant 10 hTPO, which does not suffer from the disadvantages associated with the immuno-purified native protein or with the recombinant fusion protein previously available. The present invention thus provides a number of important advances in the characterization of the human thyroid microsomal antigen, and 15 opens the way to substantial further developments in this field.

Recombinant, enzymatically-active, human thyroid peroxidase has been generated in non-thyroidal eukaryotic 20 cells. Unlike bacterial fusion proteins previously reported, the conformation of this protein is not encumbered by the  $\beta$ -galactosidase fusion partner. Furthermore, unlike the bacterially-produced protein, the TPO is glycosylated. The demonstration of functional TPO activity indicates unequivocally that the cDNA previously cloned (Magnusson, 25 R.P., *et al.*, *J. Biol. Chem.* **262**:13885-13888 (1987); Magnusson, R.P., *et al.*, *Mol. Endocrinol.* **1**:856-861 (1987); Libert, R., *et al.*, *EMBO J.* **6**:4193-4176 (1987); Kimura, S., *et al.*, *Proc. Natl. Acad. Sci. USA* **84**:5555-5559 (1987)), is indeed TPO.

30 The present invention also provides for the identification of the  $\beta$ -cell epitope on thyroid peroxidase associated with autoimmune thyroid disease. In addition, this aspect of the invention provides a method for identifying the

molecular interaction responsible for the  $\beta$ -cell recognition of thyroid peroxidase.

Experiments using the recombinant hTPO of the invention expressed in a non-thyroidal eukaryotic cell prove that TPO, independent of any other potential thyroid antigen, is a major autoantigen in Hashimoto's thyroiditis. Thus, all 36 Hashimoto's patient sera tested reacted specifically with recombinant hTPO in an approximately quantitative manner as demonstrated by Western blot analysis. While previous immunological studies strongly suggested that antimicrosomal antibodies react with hTPO (Czarnocka, B., FEBS Letters 109:147-152 (1985); Portmann, L., et al., J. Clin. Endocrinol. Metab. 61:1001-1003 (1985); Kotani, T., et al., J. Clin. Endocrinol. Metab. 62:928-933 (1986); Mariotti, S., et al., J. Clin. Endocrinol. Metab. 65:987-993 (1987)), it had been difficult to exclude the possibility of contamination of the immunopurified hTPO antigen by other, unidentified, thyroid antigens. The only thyroidal (or, indeed, human) protein produced by, or found in, the CHO-TPO cells of the present invention is hTPO. Even though human sera from both normal subjects and patients with Hashimoto's thyroiditis contain antibodies that react with some antigen(s) of untransfected CHO cells, only the Hashimoto's patient sera react with the recombinant hTPO.

The present invention also sheds light on previous observations that the microsomal antigen appeared as a doublet when analyzed by polyacrylamide gel electrophoresis (PAGE) and Western blot (Portmann, L., et al., J. Clin. Invest. 81:1217-1224 (1988); Hamada, N., et al., J. Clin. Endocrinol. Metab. 61:120-128 (1985); Hamada, N., et al., J. Clin. Invest. 79:819-825 (1987)). It was not known whether the doublet represented two separate proteins or the partial degradative product of a single protein. Kimura et al. observed two forms of hTPO mRNA and cDNA, and suggested the possibility of

alternate splicing of the initial TPO transcripts (Kimura, S., et al., Proc. Natl. Acad. Sci. USA 84:5555-5559 (1987)). Nagayama et al. reported the existence of four different forms of hTPO mRNA transcripts in cultured Graves' thyroid cells after TSH stimulation (Nagayama, Y., et al., International Thyroid Symposium, Tokyo, Japan, Abstract #42 (1988)). The present discovery of a doublet as the product of a single, intron-less, hTPO gene argues strongly against the likelihood of alternate splicing.

The apparent conversion of the doublet to a single band after protein reduction, reminiscent of the data of Portmann et al., with a crude human thyroid extract (Portmann, L., et al., J. Clin. Invest. 81:1217-1224 (1988)), suggests that membrane-bound hTPO is linked through disulfide bonds to another, unidentified protein. An alternate interpretation, in line with the model of Taurog et al. (Yokoyama, N., et al., Mol. Endocrinol. 2:838-844 (1988)), is that intrachain disulfide bonds within TPO may alter the gel migratory behavior of TPO, resulting in the appearance of multiple forms. In contrast to observations of human thyroid microsomes in which the primary antigen (under non-reducing conditions) was 107 kD in size (Hamada, N., et al., J. Clin. Endocrinol. Metab. 61:120-128 (1985)), the present inventor observed, under the same conditions, that the major immunogenic form of recombinant hTPO in transfected CHO cells is about 200 kD in mass which is converted upon reduction to a single band of about 110 kD. This difference may be related to varied expression of hTPO in different cell types (human and CHO). However, it was also reported that a 200 kD protein was produced by subjecting the extracted human thyroid microsomal 107 kD protein major band to PAGE under non-reducing conditions (Hamada, N., et al., J. Clin. Endocrinol. Metab. 61:120-128 (1985)). Also, the present finding of a diminished 110 kD signal after reduction of the recombinant

hTPO protein is in accordance with other findings using the native microsomal antigen (Gardas, A., et al., J. Endocrinol. Invest. 11:385-388 (1988); Nakajima, Y., et al., Mol. Cell. Endocrinol. 53:15-23 (1987)). Thus, in its native state, 5 human TPO exists either as a multimer or in association with another membrane protein of similar size. Epitope recognition by autoantibodies may be conformation-dependent.

The derived amino acid sequence of hTPO suggested to the present inventor the presence in recombinant full-length hTPO 10 and thus, in naturally-occurring hTPO, of a signal peptide, as well as a putative hydrophobic membrane-spanning region (transmembrane domain) at the carboxyl terminus of the protein (amino acid residues 846-870) (Magnusson, R.P., et al., J. Biol. Chem. 262:13885-13888 (1987); Magnusson, R.P., et al., 15 Mol. Endocrinol. 1:856-861 (1987); Kimura, S., et al., Proc. Natl. Acad. Sci. USA 84:5555-5559 (1987); Libert, F., et al., Nucl. Acids Res. 15:6735 (1987)). Naturally-occurring hTPO has been shown to be a thyroidal cell surface protein. Recombinant, enzymatically active hTPO is also cell membrane-associated in stably transfected non-thyroidal eukaryotic 20 cells (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)).

While not intending to be bound by a particular theory, the present inventor hypothesized that the signal peptide 25 directs the human TPO through the cell membrane, but that the hydrophobic region of hTPO becomes embedded in the cell membrane, thereby preventing secretion from the cell.

There has heretofore been no functional proof that the hTPO hydrophobic region 846-870 corresponds to a transmembrane 30 domain. The present invention demonstrates the existence of a transmembrane domain in hTPO, and that hTPO is predominantly an enzyme with an extracellular orientation. The insertion, by site-directed mutagenesis, of a stop codon immediately upstream of this putative transmembrane domain converts hTPO

into a secreted protein that is enzymatically active and immunologically intact. By introducing the stop codon, the hTPO was truncated by 85 residues, removing the carboxyl terminus (933 amino acids). Mutated hTPO cDNA, inserted 5 into a eukaryotic expression vector, was stably transfected into CHO cells. Immunoprecipitation and PAGE of cellular  $^{35}\text{S}$ -methionine-labeled proteins with Hashimoto's patient serum revealed a 105-101 kD doublet. In contrast, cells transfected with wild-type hTPO yielded a 112-105 kD doublet.

10 In pulse-chase experiments, CHO cells expressing the truncated hTPO protein secreted immunoprecipitable TPO into the culture medium after 4 hours of chase, with levels accumulating progressively over a 24 hour period. In contrast, CHO cells expressing wild-type hTPO released no immunoprecipitable TPO into the culture medium. The secreted, truncated form of hTPO appeared as a single band of lesser 15 electrophoretic mobility, as opposed to the doublet expressed within cells. TPO enzymatic activity was present in conditioned medium from CHO cells transfected with the mutated hTPO, but was absent in conditioned medium from cells 20 expressing wild-type hTPO. The stability of the mutated protein appeared similar to that of wild-type hTPO.

The secreted form of hTPO can be used to generate large amounts of soluble TPO protein for use in structural and 25 immunological studies, as well as for diagnostic uses.

Thus, in one embodiment, there is provided according to the invention recombinant, enzymatically active, TPO, or a functional or chemical derivative thereof.

In another embodiment is provided hTPO produced by non-thyroidal eukaryotic cells.

In another embodiment there is provided according to the invention recombinant hTPO that is enzymatically active, immunologically intact and secretable, or a functional or chemical derivative thereof.

Yet another embodiment of the invention comprises a plasmid selected from the group consisting of pECE-hTPO, pHTPO(M1)-ECE-SV2-DHFR, pHTPO-DHFR-2B, pHTPO-DHFR-4C and pHTPO-DHFR-4C-MTX.

5        There is also provided according to the invention a non-thyroidal eukaryotic cell transformed with any of these plasmids, as well as methods of producing hTPO comprising culturing the transformed cell under conditions allowing expression of the hTPO and recovering the hTPO.

10      In yet another embodiment, the invention provides for an antibody against the hTPO of the invention.

15      Further, a method of detecting hTPO in a sample is provided according to the present invention, comprising contacting the sample with an antibody against full-length recombinant hTPO or an antibody against a secretable hTPO, wherein the antibody is detectably labeled, so as to form a complex between the hTPO in the sample and the detectably labeled antibody, and detecting the complexed or uncomplexed labeled antibody.

20      In an additional embodiment, there is provided a kit for the detection of hTPO in a sample, comprising container means comprising one or more containers, wherein one of the containers comprises detectably labeled antibody against hTPO.

25      Further, a method of detecting antibodies to hTPO in a sample is provided according to the present invention, comprising contacting the sample with full-length recombinant hTPO or secretable recombinant hTPO so as to form a complex between an hTPO-specific antibody in the sample and the recombinant hTPO, and detecting the complexed antibody. In an additional embodiment, there is provided a kit for the detection of antibodies to hTPO in a sample, comprising container means comprising one or more containers, wherein one of said containers comprises recombinant hTPO.

These and other non-limiting embodiments of the present invention will be apparent to those of skill from the following detailed description of the invention.

5

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Construction of the expression plasmid pHTPO-ECE. pHTPO-BS (upper right) was digested with Not I, the ends blunted with the Klenow fragment of DNA polymerase I, and the DNA subsequently digested with Xba I. The released Bluescript vector was further digested with Sca I to obtain good separation on agarose gel electrophoresis because of the similar size of this vector (2.95 kb) and the HTPO cDNA fragment (3.1 kb). The mammalian expression vector pECE (Ellis, L., et al., Cell 45:721-732 (1986)) (upper left) was digested with Eco RI, the ends blunted with the Klenow fragment of DNA polymerase I, and the DNA subsequently digested with Xba I. The digested pHTPO-BS and pECE fragments were then ligated using T4 DNA ligase (Maniatis, T., et al., Molecular Biology: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)). The resulting plasmid, pHTPO-ECE (bottom), was transfected into competent XL1-Blue cells (Stratagene, San Diego, CA).

Figure 2. Fluorescence-activated cell sorter (FACS) analysis of CHO cells transfected with pHTPO-ECE. CHO-HTPO12b cells were processed as described herein.

Panel A: Cells exposed to phycoerythrin (PE)-labeled second antibody alone, without prior exposure to human serum.

Panel B: Cells incubated in serum (1:100) from a patient with Hashimoto's thyroiditis (ELISA value of 1.779) without subsequent incubation in PE-labeled second antibody.

Panel C: Cells sequentially incubated in the Hashimoto's serum described in panel B and in PE-labeled second antibody.

Panel D: As in panel C, except that serum from a normal individual, lacking antimicrosomal antibodies, was used.

5 Panels E and F: The same data as in panels C and D plotted to show the forward scatter. These data indicate that the relative sizes of the cell populations reacting with the normal and the Hashimoto's sera are the same.

Figure 3. Linear regression analysis of ELISAs using antibodies against human thyroidal microsomes or against recombinant human TPO.

10 Figure 4. Linear regression analysis of ELISAs using antibodies against human thyroidal microsomes or against recombinant human TPO, 1/1000 dilution. "Cardiff" refers to the source of the microsomal antigen of both Figures 3 and 4.

15 Figure 5. Relative TPO activities observed in CHO cells transfected with pECE-HTPO, pHPTPO-DHFR-2B and pHPTPO-DHFR-4C, shown plotted against methotrexate concentration.

20 Figure 6. Nucleotide sequence of human TPO gene after site-directed mutagenesis. The mutations incorporated two stop codons, as well as an EcoR1 site for confirmation, in the region immediately upstream from the transmembrane region of the human TPO gene.

Figure 7. cDNA sequence and derived amino acid sequence of human thyroid peroxidase (Magnusson, R.P., *et al.*, *Mol. Endocrinol.*, 1:856-861 (1987)).

25 Figure 8. Schematic diagram showing the expression plasmid pHPTPO(M1)-ECE-SV2-DHFR.

Figure 9. Construction of the plasmid pHPTPO(M1)-ECE-SV2-DHFR.

30 Figure 10. Comparison of 51 sera, selected to provide a spectrum of anti-MSA levels, in terms of their reactivity with Graves' thyroid microsomes and recombinant, enzymatically-active human TPO generated in non-thyroidal eukaryotic cells. The anti-MSA assay data are expressed as an ELISA index, relative to a standard serum. Data for the anti-hTPO antibody

assay are expressed as absolute O.D. units, normalized to a blank well value of 0.000. (A) serum dilution 1/100 (sera from four normal patients are enclosed within the rectangle); (B) serum dilution 1/1,000; (C) serum dilution 1/10,000.

5        Figure 11. Two sera (#11 and 27) reacting discrepantly with human thyroid microsomes (A) and recombinant hTPO (B) are reacting with an antigen other than hTPO in panel A at standard (1/100) serum dilution. Dilution curves are also shown for two other sera (#12 and 28) with similar anti-MSA activity at standard serum dilution.

10        Figure 12. Intra-assay variability of anti-hTPO antibody ELISA at standard (1/100) serum dilution. Mean  $\pm$  standard deviation of 10 iterations of anti-hTPO antibody ELISA results for three autoimmune sera selected to represent low, medium, 15 and high autoantibody levels.

15        Figure 13. Confirmation, by nucleotide sequencing, of the mutations introduced into hTPO by site-directed mutagenesis. The nucleotide positions referred to correspond to those reported for human TPO (Rousset, B., et al., Clin. Exp. Immunol. **52**:325-332 (1983)). TGA (2629-2631 bp) and TAG (2641-2643 bp) stop codons, as well as the EcoR1 site, in the mutated hTPO-M1 are shown on the right. The nucleotide sequence of wild-type hTPO is shown on the left.

20        Figure 14. (A) Immunoprecipitation of mutated hTPO in different clones of transfected CHO cells. CHO - non-transfected CHO cells; CHO-TPO - CHO cells transfected with wild type hTPO; CHO-TPO-M1-POOLED - pooled colonies of CHO cells transfected with the mutated form of hTPO; CHO-TPO-M1-D through K - individual colonies of CHO cells, transfected with mutated hTPO, that were selected with cloning cylinders and then expanded. Cells were radiolabeled with  $^{35}$ S-methionine and immunoprecipitated with Hashimoto's thyroiditis serum containing high anti-hTPO antibody levels.

(B) Immunoprecipitation of mutated hTPO from clones of CHO-TPO-M1-K cells generated by limiting dilution. Immunoprecipitations were performed with serum from a patient with Hashimoto's thyroiditis with high anti-hTPO antibody levels. The specificity of the immunoprecipitation is shown by the inability of serum from a normal individual (CON) to precipitate the 105-101 kD doublet.

Figure 15. Biosynthesis and processing of TPO. Immuno-precipitation studies were performed with CHO cells expressing wild-type hTPO (upper panel), and with CHO cells transfected with the mutated form of hTPO (lower panel). Pulse for 4 h (0 hours of chase) with  $^{35}$ S-methionine was followed by chase with unlabeled methionine for the indicated periods of time. Immunoprecipitations were then performed on both cell lysates and conditioned media, as indicated.

Figure 16. Human TPO enzymatic activity in the medium of CHO cells after transfection with wild-type hTPO (cell line CHO-TPO 12g) (Kotani, T., et al., *J. Clin. Endocrinol. Metab.* **62**:928-933 (1986)) and CHO cells transfected with the mutated form of hTPO (CHO-TPO-M1-K1). Media were collected after 3 days of culture. TPO enzymatic activity in the media was measured by the guaiacol assay. The time course shown refers to the accumulation of oxidized guaiacol substrate in the assay, and not to the kinetics of enzyme secretion into the medium.

Figure 17. (A) T cell clones from the thyroid infiltrate in Graves' disease, expanded in the absence of antigen, recognize recombinant TPO. Clone + autologous irradiated PBL - black bars; clone + PBL + control (untransfected) CHO microsomes - striped bars; clone + PBL + CHO microsomes transfected with TPO - grey bars. Results are expressed as mean cpm of [ $^3$ H]thymidine incorporation from triplicate cultures. Error bars indicate standard errors of the mean

(S.E.M.). Similar results were obtained in three or more replicate experiments.

(B) Peripheral blood lymphocytes from both patients and normal subjects proliferate in response to both control and TPO-transfected microsomes. PBL alone - black bars; PBL + control microsomes - striped bars; PBL + TPO transfected microsomes - grey bars. Results are expressed as mean cpm [<sup>3</sup>H]thymidine incorporation of triplicate cultures (Error bars indicate S.E.M.) 81 - patient from whom T cell clones in Fig. 17A were derived; RG - another female with Graves' disease; KH - normal control female. Similar results have been obtained from other individuals in separate experiments.

Figure 18. Determination of the epitope for the anti-microsomal/TPO monoclonal antibody 20.10. The nucleotide sequences of the 5'- and 3'-ends were determined for 14 clones selected from the hTPO cDNA fragment library. These boundaries are annotated by the numbers assigned to the nucleotides in hTPO previously reported (Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861 (1987)). The smallest region of overlap between all 14 clones is from 881-927 b.p. The first two nucleotides in this span do not constitute a complete codon, so the epitope area can be defined as between 883-927 b.p., corresponding to the derived amino acid sequence shown.

Figure 19. Determination of the epitope recognized by TPO MAb 47. The nucleotide sequences of the 5'- and 3'-prime ends were determined for 18 clones in the TPO cDNA fragment library (see Materials and Methods) recognized by MAb 47. The smallest region of overlap between all 18 clones is from 2219-2247 basepairs in the human TPO cDNA sequence, coding for the indicated amino acids.

Figure 20. Western blot analysis of human TPO, using TPO MAb. Recombinant human TPO expressed in Chinese hamster

ovary cells was used as antigen under denaturing and reducing conditions (see Materials and Methods). After polyacrylamide gel electrophoresis and transfer to the membranes, the membranes were probed with the indicated antibodies. TPO MAb 5 1, 2, 9, 15, 18, 24, 30, 40, 47, 53, 59, 60 and 64 are mouse MAbs generated against native undenatured human TPO (Ruf, J., et al., Endocrinology 125:1211-8 (1989)). The controls (Con) are mouse MAbs raised against denatured human TPO (Portmann, L., et al., J. Clin. Invest. 81:1217-1224 (1988)) (A and B) 10 and control mouse ascitic fluid (C). The sizes of the mol wt markers are shown on the left, and that of recombinant human TPO is indicated by the arrow.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

15

In the following description, reference will be made to various methodologies known to those of skill in the art of molecular biology and immunology. Publications and other materials setting forth such known methodologies to which reference is made are incorporated herein by reference in their entireties as though set forth in full.

20

Standard reference works setting forth the general principles of recombinant DNA technology include Watson, J.D. et al., Molecular Biology of the Gene, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., publisher, Menlo Park, CA (1987); Darnell, J.E. et al., Molecular Cell Biology, Scientific American Books, Inc., publisher, New York, N.Y. (1986); Lewin, B.M., Genes II, John Wiley & Sons, publishers, New York, N.Y. (1985); Old, R.W., et al., Principles of Gene Manipulation: An Introduction to Genetic Engineering, 2d edition, University of California Press, publisher, Berkeley, CA (1981); and Maniatis, T., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, publisher, Cold Spring Harbor, NY (1982).

By "cloning" is meant the use of in vitro recombination techniques to insert a particular gene or other DNA sequence into a vector molecule. In order to successfully clone a desired gene, it is necessary to employ methods for generating 5 DNA fragments, for joining the fragments to vector molecules, for introducing the composite DNA molecule into a host cell in which it can replicate, and for selecting the clone having the target gene from amongst the recipient host cells.

By "cDNA" is meant complementary or copy DNA produced 10 from an RNA template by the action of RNA-dependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector.

By "cDNA library" is meant a collection of recombinant 15 DNA molecules containing cDNA inserts which together comprise the entire genome of an organism. Such a cDNA library may be prepared by methods known to those of skill, and described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, supra. Generally, RNA is first isolated 20 from the cells of an organism from whose genome it is desired to clone a particular gene. Preferred for the purposes of the present invention are mammalian, and particularly human, cell lines. A presently preferred vector for this purpose is the λ-ZAP vector.

By "vector" is meant a DNA molecule, derived from a plasmid or bacteriophage, into which fragments of DNA may be inserted or cloned. A vector will contain one or more unique 25 restriction sites, and may be capable of autonomous replication in a defined host or vehicle organism such that the cloned sequence is reproducible. Thus, by "DNA expression vector" is meant any autonomous element capable of replicating in a host independently of the host's chromosome, after additional sequences of DNA have been incorporated into the

autonomous element's genome. Such DNA expression vectors include bacterial plasmids and phages.

By "substantially pure" is meant any antigen of the present invention, or any gene encoding any such antigen, which is essentially free of other antigens or genes, respectively, or of other contaminants with which it might normally be found in nature, and as such exists in a form not found in nature. By "functional derivative" is meant the "fragments," "variants," "analogs," or "chemical derivatives" of a molecule. A "fragment" of a molecule, such as any of the cDNA sequences of the present invention, is meant to refer to any nucleotide subset of the molecule. A "variant" of such molecule is meant to refer to a naturally occurring molecule substantially similar to either the entire molecule, or a fragment thereof. An "analog" of a molecule is meant to refer to a non-natural molecule substantially similar to either the entire molecule or a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if the sequence of amino acids in both molecules is substantially the same. Substantially similar amino acid molecules will possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants as that term is used herein even if one of the molecules contains additional amino acid residues not found in the other, or if the sequence of amino acid residues is not identical. As used herein, a molecule is said to be a "chemical derivative" of another molecule when it contains additional chemical moieties not normally a part of the molecule. Such moieties may improve the molecule's solubility, absorption, biological half life, etc. The moieties may alternatively decrease the toxicity of the molecule, eliminate or attenuate any undesirable side effect of the molecule, etc. Moieties capable of mediating such effects are disclosed, for example, in Remington's

Pharmaceutical Sciences, 16th ed., Mack Publishing Co.,  
Easton, Penn. (1980).

Similarly, a "functional derivative" of a gene of the  
human TPO antigen of the present invention is meant to include  
5 "fragments," "variants," or "analogues" of the gene, which may  
be "substantially similar" in nucleotide sequence, and which  
encode a molecule possessing similar activity.

A DNA sequence encoding the human thyroid peroxidase of  
the present invention, or its functional derivatives, may be  
10 recombined with vector DNA in accordance with conventional  
techniques, including blunt-ended or staggered-ended termini  
for ligation, restriction enzyme digestion to provide  
appropriate termini, filling in of cohesive ends as  
appropriate, alkaline phosphatase treatment to avoid  
15 undesirable joining, and ligation with appropriate ligases.  
Techniques for such manipulations are disclosed by Maniatis,  
T., et al., supra, and are well known in the art.

By "secretion" of recombinant hTPO for the purposes of  
the present invention, it is meant that the recombinant hTPO  
20 expressed by a host cell is directed through and dissociated  
from the host cell membrane.

A nucleic acid molecule, such as DNA, is said to be  
"capable of expressing" a polypeptide if it contains  
nucleotide sequences which contain transcriptional and  
25 translational regulatory information and such sequences are  
"operably linked" to nucleotide sequences which encode the  
polypeptide. An operable linkage is a linkage in which the  
regulatory DNA sequences and the DNA sequence sought to be  
expressed are connected in such a way as to permit gene  
30 expression. The precise nature of the regulatory regions  
needed for gene expression may vary from organism to organism,  
but shall in general include a promoter region which, in  
prokaryotes, contains both the promoter (which directs the  
initiation of RNA transcription) as well as the DNA sequences

which, when transcribed into RNA, will signal the initiation of protein synthesis. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the gene sequence coding for the protein may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence coding for the protein, the transcriptional termination signals may be provided. Where the transcriptional termination signals are not satisfactorily functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and a human thyroid peroxidase encoding sequence) are said to be operably linked if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of the thyroid peroxidase gene sequence, or (3) interfere with the ability of the thyroid peroxidase gene sequence to be transcribed by the promoter region sequence. A promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express the protein, transcriptional and translational signals recognized by an appropriate host are necessary.

The present invention encompasses the expression of the human thyroid peroxidase protein (or a functional derivative thereof) in either prokaryotic or eukaryotic cells, although eukaryotic (and, particularly, non-thyroidal eukaryotic) expression is preferred.

Preferred prokaryotic hosts include bacteria such as E. coli, Bacillus, Streptomyces, Pseudomonas, Salmonella, Serratia, etc. The most preferred prokaryotic host is E. coli. Other enterobacteria such as Salmonella typhimurium or 5 Serratia marcescens, and various Pseudomonas species may also be utilized. Under such conditions, the protein may not be glycosylated. The prokaryotic host must be compatible with the replicon and control sequences in the expression plasmid.

To express the human thyroid peroxidase protein (or a functional derivative thereof) in a prokaryotic cell (such as, 10 for example, E. coli, B. subtilis, Pseudomonas, Streptomyces, etc.), it is necessary to operably link the human TPO encoding sequence to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable 15 (i.e., inducible or derepressible). Examples of constitutive promoters include the int promoter of bacteriophage  $\lambda$ , the bla promoter of the  $\beta$ -lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene of pBR325, etc. Examples of inducible prokaryotic promoters 20 include the major right and left promoters of bacteriophage  $\lambda$  ( $P_L$  and  $P_R$ ), the trp, recA, lacZ, lacI, and gal promoters of E. coli, the  $\alpha$ -amylase (Ulmanen, I., et al., J. Bacteriol. 162:176-182 (1985)) and the  $\sigma$ -28-specific promoters of B. subtilis (Gilman, M.Z., et al., Gene 32:11-20 (1984)), the 25 promoters of the bacteriophages of Bacillus (Gryczan, T.J., In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY (1982)), and Streptomyces promoters (Ward, J.M., et al., Mol. Gen. Genet. 203:468-478 (1986)). Prokaryotic promoters are reviewed by Glick, B.R., (J. Ind. Microbiol. 1:277-282 (1987)); Cenatiempo, Y. (Biochimie 68:505-516 30 (1986)); and Gottesman, S. (Ann. Rev. Genet. 18:415-442 (1984)).

Proper expression in a prokaryotic cell also requires the presence of a ribosome binding site upstream of the gene-

encoding sequence. Such ribosome binding sites are disclosed, for example, by Gold, L., et al. (Ann. Rev. Microbiol. **35**:365-404 (1981)).

5        Most preferred hosts are eukaryotic hosts including yeast, insects, fungi, and mammalian cells either in vivo, or in tissue culture. Mammalian cells provide post-translational modifications to protein molecules including correct folding or glycosylation at correct sites. Mammalian cells which may be useful as hosts include cells of fibroblast origin such as  
10      VERO or CHO-K1, or cells of lymphoid origin, such as the hybridoma SP2/O-AG14 or the myeloma P3x63Sg8, and their derivatives. CHO cells are presently preferred mammalian host cells. COS cells also are convenient eukaryotic hosts for  
15      human thyroid peroxidase expression, as well as for study of the regulation of human thyroid peroxidase expression.

For a mammalian cell host, many possible vector systems are available for the expression of human TPO. A wide variety of transcriptional and translational regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, Simian virus, or the like, where the regulatory signals are associated with a particular gene which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, etc., may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical regulation, e.g., metabolite.  
20  
25  
30

Yeast provides substantial advantages in that it can also carry out post-translational peptide modifications including

glycosylation. A number of recombinant DNA strategies exist which utilize strong promoter sequences and high copy number of plasmids which can be utilized for production of the desired proteins in yeast. Yeast recognizes leader sequences 5 on cloned mammalian gene products and secretes peptides bearing leader sequences (i.e., pre-peptides).

Further, by use of, for example, the yeast ubiquitin hydrolase system, in vivo synthesis of ubiquitin-human TPO fusion proteins may be accomplished. The fusion proteins so 10 produced may be processed in vivo or purified and processed in vitro, allowing synthesis of the human TPO protein with a specified amino terminus sequence. Moreover, problems associated with retention of initiation codon-derived 15 methionine residues in direct yeast (or bacterial) expression may be avoided. Sabin *et al.*, Bio/Technol. 7(7): 705-709 (1989); Miller *et al.*, Bio/Technol. 7(7): 698-704 (1989).

Any of a series of yeast gene expression systems incorporating promoter and termination elements from the actively expressed genes coding for glycolytic enzymes 20 produced in large quantities when yeast are grown in mediums rich in glucose can be utilized. Known glycolytic genes can also provide very efficient transcriptional control signals. For example, the promoter and terminator signals of the phosphoglycerate kinase gene can be utilized.

Production of human TPO or functional derivatives thereof 25 in insects can be achieved, for example, by infecting the insect host with a baculovirus engineered to express human TPO by methods known to those of skill. Thus, in one embodiment, sequences encoding human TPO may be operably linked to the regulatory regions of the viral polyhedrin protein (Jasny, 30 Science 238: 1653 (1987)). Infected with the recombinant baculovirus, cultured insect cells, or the live insects themselves, can produce the human TPO protein in amounts as great as 20 to 50% of total protein production. When live

insects are to be used, caterpillars are presently preferred hosts for large scale human TPO production according to the invention.

As discussed above, expression of the human thyroid peroxidase protein in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer, D., et al., J. Mol. Appl. Gen. 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, S., Cell 31:355-365 (1982)); the SV40 early promoter (Benoist, C., et al., Nature (London) 290:304-310 (1981)); the yeast gal4 gene promoter (Johnston, S.A., et al., Proc. Natl. Acad. Sci. (USA) 79:6971-6975 (1982); Silver, P.A., et al., Proc. Natl. Acad. Sci. (USA) 81:5951-5955 (1984)). Of these, presently the most preferred is the SV40 promoter.

As is widely known, translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the human TPO protein (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as human TPO encoding DNA sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the human TPO encoding sequence).

The human TPO encoding sequence and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a non-replicating DNA (or RNA) molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such

molecules are incapable of autonomous replication, the expression of the human TPO protein may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced sequence into the host chromosome.

In one embodiment, a vector is employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotropic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of single chain binding protein mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama, H., Mol. Cel. Biol. 3:280 (1983).

In a preferred embodiment, the introduced sequence will be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species. Preferred prokaryotic vectors include plasmids such as those capable of

replication in E. coli (such as, for example, pBR322, ColE1, pSC101, pACYC 184,  $\pi$ VX. Such plasmids are, for example, disclosed by Maniatis, T., et al. (In: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, NY (1982)). Bacillus plasmids include pC194, pC221, pT127, etc. Such plasmids are disclosed by Gryczan, T. (In: The Molecular Biology of the Bacilli, Academic Press, NY (1982), pp. 307-329). Suitable Streptomyces plasmids include pIJ101 (Kendall, K.J., et al., J. Bacteriol. 169:4177-4183 (1987)), and streptomyces bacteriophages such as  $\phi$ C31 (Chater, K.F., et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kaido, Budapest, Hungary (1986), pp. 45-54). Pseudomonas plasmids are reviewed by John, J.F., et al. (Rev. Infect. Dis. 8:693-704 (1986)), and Izaki, K. (Jpn. J. Bacteriol. 33:729-742 (1978)).

Preferred eukaryotic plasmids include BPV, vaccinia, SV40, 2-micron circle, etc., or their derivatives. Such plasmids are well known in the art (Botstein, D., et al., Miami Wntr. Symp. 19:265-274 (1982); Broach, J.R., In: The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470 (1981); Broach, J.R., Cell 28:203-204 (1982); Bollon, D.P., et al., J. Clin. Hematol. Oncol. 10:39-48 (1980); Maniatis, T., In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Expression, Academic Press, NY, pp. 563-608 (1980).

Once the vector or DNA sequence containing the construct(s) has been prepared for expression, the vector or DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means, including such biochemical means as transformation, transfection, conjugation, protoplast fusion, calcium phosphate-precipitation, and application with polycations such as diethylaminoethyl (DEAE) dextran, and such mechanical means as

electroporation, direct microinjection, and microprojectile (biostatic) bombardment (Johnston *et al.*, Science 240(4858): 1538 (1988)), etc.

5 After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene sequence(s) results in the production of the human TPO protein, or in the production of a fragment of this protein. This can take place in the transformed cells as such, or  
10 following the induction of these cells to differentiate.

The expressed protein may be isolated and purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like. For example, the cells may be  
15 collected by centrifugation, or with suitable buffers, lysed, and the protein isolated by column chromatography, for example, on DEAE-cellulose, phosphocellulose, polyribocytidyllic acid-agarose, hydroxyapatite or by electrophoresis or immunoprecipitation. Alternatively, the human TPO or  
20 functional derivative thereof may be isolated by the use of anti-human TPO antibodies. Such antibodies may be obtained by well-known methods, some of which as mentioned hereinafter.

#### ANTIBODIES SPECIFIC FOR hTPO

25 The term "antibody" (Ab) or "monoclonal antibody" (mAb) as used herein is meant to include intact molecules as well as fragments thereof (such as, for example, Fab and F(ab')<sub>2</sub> fragments) which are capable of binding an antigen. Fab and F(ab')<sub>2</sub> fragments lack the Fc fragment of intact antibody,  
30 clear more rapidly from the circulation, and may have less non-specific tissue binding of an intact antibody (*Wahl et al., J. Nucl. Med. 24:316-325 (1983)*).

Antibodies according to the present invention may be prepared by any of a variety of methods. For example, cells

expressing the human TPO protein, or a functional derivative thereof, can be administered to an animal in order to induce the production of sera containing polyclonal antibodies that are capable of binding human TPO.

5       In a preferred method, antibodies according to the present invention are mAbs. Such mAbs can be prepared using hybridoma technology (Kohler *et al.*, Nature 256:495 (1975); Kohler *et al.*, Eur. J. Immunol. 6:511 (1976); Kohler *et al.*, Eur. J. Immunol. 6:292 (1976); Hammerling *et al.*, In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures involve immunizing an animal with human TPO antigen. The splenocytes of such animals are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands, J.R., *et al.* (Gastroenterology 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the human TPO antigen.

10       Antibodies according to the present invention also may be polyclonal, or, preferably, region specific polyclonal antibodies. Region specific polyclonal antibodies and methods of using them are described in co-pending U.S. application Serial Number 06/731,470, filed 07 May 1985, the specification of which is incorporated herein by reference as though set forth in full.

15       Antibodies against human TPO according to the present invention are well suited for use in standard immunodiagnostic assays known in the art, including such immunometric or "sandwich" assays as the forward sandwich, reverse sandwich, and simultaneous sandwich assays. The antibodies may be used in any number of combinations as may be determined by those of

skill without undue experimentation to effect immunoassays of acceptable specificity, sensitivity, and accuracy for the human TPO antigen or equivalents thereof.

Standard reference works setting forth general principles of immunology include Roitt, I., Essential Immunology, Sixth Ed., Blackwell Scientific Publications, Publisher, Oxford (1988); Kimball, J. W., Introduction to Immunology, Second Ed., Macmillan Publishing Co., Publisher, New York (1986); Roitt, I., et al., Immunology, Gower Medical Publishing Ltd., Publisher, London, (1985); Campbell, A., "Monoclonal Antibody Technology," in, Burdon, R., et al., eds., Laboratory Techniques in Biochemistry and Molecular Biology, Volume 13, Elsevier, Publisher, Amsterdam (1984); Klein, J., Immunology: The Science of Self-Nonself Discrimination, John Wiley & Sons, Publisher, New York (1982); and Kennett, R., et al., eds., Monoclonal Antibodies. Hybridoma: A New Dimension in Biological Analyses, Plenum Press, Publisher, New York (1980).

By "detecting" it is intended to include determining the presence or absence of a substance or quantifying the amount of a substance. The term thus refers to the use of the materials, compositions, and methods of the present invention for qualitative and quantitative determinations.

The isolation of other hybridomas secreting mAbs of the same specificity as those described herein can be accomplished by the technique of anti-idiotypic screening. Potocnjak, et al., Science 215:1637 (1982). Briefly, an anti-idiotypic (anti-Id) antibody is an antibody which recognizes unique determinants generally associated with the antigen-binding site of an antibody. An Id antibody can be prepared by immunizing an animal of the same species and genetic type (e.g. mouse strain) as the source of the mAb with the mAb to which an anti-Id is being prepared. The immunized animal will recognize and respond to the idiotypic determinants of the

immunizing antibody by producing an antibody to these idiotypic determinants (the anti-Id antibody).

By using an anti-Id antibody which is specific for idiotypic determinants on a given mAb, it is then possible to  
5 identify other B cell or hybridoma clones sharing that idiotype. Idiotypic identity between the antibody product of two clones makes it highly probable that the antibody products of the two clones recognize the same antigenic epitopes.

The anti-Id antibody may also be used as an "immunogen"  
10 to induce an immune response in yet another animal, producing a so-called anti-anti-Id antibody. The anti-anti-Id may be epitopically identical to the original mAb which induced the anti-Id.

Thus, by using antibodies to the idiotypic determinants  
15 of a mAb, it is possible to identify other clones expressing antibodies of identical specificity.

Accordingly, mAbs generated against the hTPO antigen may be used to induce anti-Id antibodies in suitable animals, such as BALB/c mice. Spleen cells from such immunized mice are  
20 used to produce anti-Id hybridomas secreting anti-Id mAbs. Further, the anti-Id mAbs can be coupled to a carrier such as keyhole limpet hemocyanin (KLH) and used to immunize additional BALB/c mice. Sera from these mice will contain anti-anti-Id antibodies that have the binding properties of  
25 the original mAb specific for the hPTO epitope. The anti-Id mAbs thus have their own idiotypic epitopes, or "idiotopes" structurally similar to the epitope being evaluated, such as hTPO.

For replication, the hybridoma cells of this invention  
30 may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo production makes this the presently preferred method of production. Briefly, cells from the individual hybridomas are injected intraperitoneally into pristane-primed BALB/c mice to produce ascites fluid

containing high concentrations of the desired mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using column chromatography methods well known to those of skill in the art.

5       Antibodies according to the present invention are particularly suited for use in immunoassays wherein they may be utilized in liquid phase or bound to a solid phase carrier. In addition, the antibodies in these immunoassays can be detectably labeled in various ways.

10      There are many different labels and methods of labeling known in the art. Examples of the types of labels which can be used in the present invention include, but are not limited to, enzymes, radioisotopes, fluorescent compounds, chemiluminescent compounds, bioluminescent compounds and metal chelates. Those of ordinary skill in the art will know of other suitable labels for binding to antibodies, or will be able to ascertain the same by the use of routine experimentation. Furthermore, the binding of these labels to antibodies can be accomplished using standard techniques commonly known to those of ordinary skill in the art.

15      One of the ways in which antibodies according to the present invention can be detectably labeled is by linking the antibody to an enzyme. This enzyme, in turn, when later exposed to its substrate, will react with the substrate in such a manner as to produce a chemical moiety which can be detected as, for example, by spectrophotometric or fluorometric means. Examples of enzymes which can be used to detectably label antibodies include malate dehydrogenase, staphylococcal nuclease, delta-V-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate dehydrogenase, triose phosphate isomerase, biotin-avidin peroxidase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease,

catalase, glucose-VI-phosphate dehydrogenase, glucoamylase and acetylcholine esterase.

The presence of detectably labeled antibodies also can be detected by labeling the antibodies with a radioactive isotope which then can be determined by such means as the use of a gamma counter or a scintillation counter. Isotopes which are particularly useful for the purpose of the present invention are  $^3\text{H}$ ,  $^{125}\text{I}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{51}\text{Cr}$ ,  $^{36}\text{Cl}$ ,  $^{57}\text{Co}$ ,  $^{58}\text{Co}$ ,  $^{59}\text{Fe}$  and  $^{75}\text{Se}$ .

It is also possible to detect the binding of detectably labeled antibodies by labeling the antibodies with a fluorescent compound. When a fluorescently labeled antibody is exposed to light of the proper wave length, its presence then can be detected due to the fluorescence of the dye. Among the most commonly used fluorescent labeling compounds are fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The antibodies of the invention also can be detectably labeled using fluorescent emitting metals such as  $^{152}\text{Eu}$ , or others of the lanthanide series. These metals can be attached to the antibody molecule using such metal chelating groups as diethylenetriaminepentaacetic acid (DTPA) or ethylenediamine-tetraacetic acid (EDTA).

Antibodies also can be detectably labeled by coupling them to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting the presence of luminescence that arises during the course of the chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salts, oxalate ester, and dioxetane.

Likewise, a bioluminescent compound may be used to label the antibodies according to the present invention. Biolumi-

5        nescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent antibody is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling include luciferin, luciferase and aequorin.

10      The antibodies and substantially purified antigen of the present invention are ideally suited for the preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement therewith one or more container means such as vials, tubes and the like, each of said container means comprising the separate elements of the assay to be used.

15      The types of assays which can be incorporated in kit form are many, and include, for example, competitive and non-competitive assays. Typical examples of assays which can utilize the antibodies of the invention are radioimmunoassays (RIA), enzyme immunoassays (EIA), enzyme-linked immunosorbent assays (ELISA), and immunometric, or sandwich, immunoassays.

20      By the term "immunometric assay" or "sandwich immunoassay," it is meant to include simultaneous sandwich, forward sandwich and reverse sandwich immunoassays. These terms are well understood by those skilled in the art. Those of skill will also appreciate that antibodies according to the present invention will be useful in other variations and forms of assays which are presently known or which may be developed in the future. These are intended to be included within the scope of the present invention.

25      Forward sandwich assays are described, for example, in United States Patents 3,867,517; 4,012,294 and 4,376,110. Reverse sandwich assays have been described, for example, in United States Patents 4,098,876 and 4,376,110.

30      In the preferred mode for performing the assays it is important that certain "blockers" be present in the incubation

medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific proteins, protease, or human antibodies to mouse immunoglobulins present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive or false negative results. The selection of "blockers" therefore adds substantially to the specificity of the assays described in the present invention.

It has been found that a number of nonrelevant (i.e. non-specific) antibodies of the same class or subclass (isotype) as those used in the assays (e.g. IgG<sub>1</sub>, IgG<sub>2a</sub>, IgM, etc.) can be used as "blockers." The concentration of the "blockers" (normally 1-100 µg/ml) is important, in order to maintain the proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive proteins in human serum. In addition, the buffer system containing the "blockers" needs to be optimized. Preferred buffers are those based on weak organic acids, such as imidazole, HEPES, MOPS, TES, ADA, ACES, HEPES, PIPES, TRIS, and the like, at physiological pH ranges. Somewhat less preferred buffers are inorganic buffers such as phosphate, borate or carbonate. Finally, known protease inhibitors should be added (normally at 0.01-10 µg/ml) to the buffer which contains the "blockers."

There are many solid phase immunoadsorbents which have been employed and which can be used in the present invention. Well known immunoadsorbents include glass, polystyrene, polypropylene, dextran, nylon and other materials, in the form of tubes, beads, and microtiter plates formed from or coated with such materials, and the like. The immobilized antibodies can be either covalently or physically bound to the solid phase immunoadsorbent, by techniques such as covalent bonding via an amide or ester linkage, or by adsorption. Those skilled in the art will know many other suitable solid phase

immunoadsorbents and methods for immobilizing antibodies thereon, or will be able to ascertain such, using no more than routine experimentation.

For in vivo, in vitro or in situ diagnosis, labels such as radionuclides may be bound to antibodies according to the present invention either directly or by using an intermediary functional group. An intermediary group which is often used to bind radioisotopes which exist as metallic cations to antibodies is diethylenetriaminepentaacetic acid (DTPA). Typical examples of metallic cations which are bound in this manner are:  $^{99m}\text{Tc}$ ,  $^{123}\text{I}$ ,  $^{111}\text{IN}$ ,  $^{131}\text{I}$ ,  $^{97}\text{Ru}$ ,  $^{67}\text{Cu}$ ,  $^{67}\text{Ga}$  and  $^{68}\text{Ga}$ . The antibodies of the invention can also be labeled with non-radioactive isotopes for purposes of diagnosis. Elements which are particularly useful in this manner are  $^{157}\text{Gd}$ ,  $^{55}\text{Mn}$ ,  $^{162}\text{Dy}$ ,  $^{52}\text{Cr}$  and  $^{56}\text{Fe}$ .

The hTPO-encoding DNA sequence of the present invention, or a fragment thereof, may be used as a DNA probe to isolate or detect complementary DNA sequences according to well-known hybridization methods. The human antigen genes may then be cloned and expressed in a host to give the human antigen. This human antigen may then be used in diagnostic assays for the corresponding autoantibody.

The antigen of the invention may be isolated in substantially pure form employing antibodies according to the present invention. Thus, an embodiment of the present invention provides for substantially pure hTPO, characterized in that it is recognized by and binds to the anti-hTPO antibodies of the present invention. In another embodiment, the present invention provides a method of isolating or purifying hTPO by forming a complex with one or more antibodies directed against hTPO.

The substantially pure hTPO of the present invention may in turn be used to detect or measure antibody to hTPO in a sample, such as serum or urine. Thus, one embodiment of the

present invention comprises a method of detecting the presence or amount of antibody to hTPO in a sample, comprising contacting the sample containing the antibody to hTPO with detectably labeled hTPO, and detecting the label.

5 It will be appreciated that immunoreactive fractions and immunoreactive analogs of hTPO also may be used. By the term "immunoreactive fraction" is intended any portion of the hTPO antigen which demonstrates an equivalent recognition by, or binding to, an antibody directed against hTPO. By the term 10 "immunoreactive analog" is intended a protein which differs from hTPO by one or more amino acids, but which demonstrates an equivalent recognition by, or binding to, an anti-hTPO antibody.

15 T CELLS SPECIFIC FOR TPO

Autoimmune diseases are thought to result at least in part due to persistent activation of T cells by self antigens (Janeway, C., Nature 341: 482 (1989)). In the case of autoimmune thyroiditis, as in Hashimoto's thyroiditis, such a 20 self antigen can be any epitope of TPO which is recognized by a receptor on a T cell capable of helping a B cell make an anti-TPO antibody, or a T cell involved in the autoimmune process by any other known mechanism (see below).

One approach to the treatment of autoimmune thyroid 25 diseases as contemplated by the present inventor focuses on disrupting the action of T lymphocytes involved in the disease process. T cells are readily available from the thyroid, for example in Graves' disease in the form of infiltrates extracted from thyroidectomy specimens. By studying such 30 infiltrates, it is possible to examine the antigenic specificities of T cells selected in vivo for their pathogenic relevance.

For example, the infiltrating T cells (as well as T cells present in the circulation and in lymphoid organs such as

- lymph nodes and spleen) can act as T helper (Th) cells, responding to TPO epitopes, and helping B cells make specific anti-TPO antibodies. Alternatively, or additionally, such T cells can mediate a cell-mediated immune response and act on thyroid epithelial cells either directly or via the local release of cytokines. This may lead to destruction of thyroid epithelial cells, when cytotoxic T cells specific for TPO are activated, or via an inflammatory response mediated by a different T cell class.
- Disruption of the activation or action of such T cells would serve to inhibit the production of anti-TPO antibodies, on the one hand, or of thyroid epithelium-damaging T cells on the other.
- One embodiment therefore provides peptides capable of binding to the T cell receptor (TCR) of a TPO-specific T cell. Such TPO-related peptides include at least a portion of a T cell epitope of TPO (such as the NP-7 epitope of Example XII). Useful peptides include a sequence of about 5 or more amino acids of TPO, or derivatives of such peptides, which are capable of binding to the TCR of a TPO-specific T cell. Acting as a competitive antagonist for the native autoantigen, such a peptide can inhibit antigen presentation to T cells, or other antigen-specific cell-cell (e.g., T-T or T-B) interactions in the immune system which are needed for generation of either anti-TPO antibodies or TPO-specific cell-mediated immunity. (For discussion of such peptide-based approaches to immunotherapy of autoimmune disease, see, for example: Acha-Orbea, H., et al., *Ann. Rev. Immunol.* **7**:371-405 (1989); Kumar, V., et al., *Ann. Rev. Immunol.* **7**:657-682 (1989); Urban, J.L. et al., *Cell* **54**:577-592 (1989); Wraith, D.C., et al., *Cell* **57**:709-715 (1989); Wraith, D.C., et al., *Cell* **59**:247-255 (1989); Urban, J.L., et al., *Cell* **59**:257-271 (1989); and Janeway, C.A., *Nature* **341**:482-483 (1989), all of which references are hereby incorporated by reference).

Another embodiment of the invention provides for a pharmaceutical preparation comprising the above peptides. In yet another embodiment of the invention, a method of treating autoimmune disease, including but not limited to Hashimoto's thyroiditis, is provided which comprises administering to a patient suffering from such disease a pharmaceutical preparation comprising a TPO-related peptide.

An alternate peptide-based therapeutic strategy contemplated within the scope of the present invention is directed to vaccines comprising TPO-specific T cells (Cohen, I.R., Immunol. Rev. 94:5-21 (1986); Prog. Immunol. VI:491-499 (1986); Scientific Amer. 258:52-60 (1988); Hosp. Prac. pp. 57-64 (February 15, 1989); Cohen, I.R., et al., Immunol. Today 9:332-335 (1988)) and peptides mimicking the TCR of such TPO-specific T cells (Vandenbark, A.A. et al., Nature 341:541-544 (1989); Howell, M.D. et al., Science 246:668-671 (1989)). Such preparations are administered to an individual to prevent or suppress an autoimmune response to TPO by inducing a state of "counter-autoimmunity." Such counter-autoimmunity is thought to be mediated by T cells which are specific to the TCR of the autoimmune (i.e., TPO-specific) T cell (Cohen, supra, Vandenbark et al., supra, and Sun, D. et al., Nature 332:843-845 (1988); Europ. J. Immunol. 18:1993-1999 (1988)).

The invention is therefore directed to T cells specific for TPO capable of acting as a "vaccine" and inducing a state of counter-autoimmunity. Another embodiment includes TCR-mimicking peptides of such T cells. Yet another embodiment is directed to the T cells induced by such TPO-specific T cell and TCR peptide vaccines which mediate the counter-autoimmune effects or down-regulate TPO-specific T cells. Another embodiment of the invention provides for a pharmaceutical preparation comprising such a T cell vaccine, TCR peptide, or counter-autoimmune T cell. In yet another embodiment of the

current invention, a method of treating autoimmune disease, such as Hashimoto's thyroiditis, is provided which includes the use of a pharmaceutical preparation comprising either a TPO-specific T cell vaccine, a TCR peptide vaccine, or a counter-autoimmune T cell specific for TPO-specific T cells.

An additional embodiment of the present invention is directed to a T suppressor (Ts) lymphocyte capable of interacting specifically with an anti-TPO B cell or T cell, leading to suppression of an anti-TPO immune response. Such suppression could be of TPO-specific antibody production or of TPO-specific T cell-mediated thyroid damage such as that mediated by cytotoxic T cells or in a TPO-specific delayed hypersensitivity response. Thus in one embodiment, the invention is directed to an epitope of TPO capable of inducing antigen-specific Ts cells and its use in generating Ts cells and in treating autoimmune thyroiditis. Another embodiment is a TPO-specific Ts in a pharmaceutical preparation. Yet another embodiment is directed to a method of treating autoimmune thyroiditis, such as Hashimoto's disease, comprising administering a pharmaceutical preparation comprising a TPO epitope capable of inducing Ts cells. An additional embodiment is a method of treating autoimmune thyroiditis by administering a pharmaceutical preparation comprising TPO-specific Ts cells capable of suppressing an anti-TPO response. For a discussion of suppressor cells, see, for example, Green, D., et al., *Ann. Rev. Immunol.* 1: 439 (1983) and Benacerraf, B., In: The Biology of Immunologic Disease, HP Publishing Co., Inc., NY, pp. 49-62 (1983).

The present invention allows the determination of the T cell epitope or epitopes of TPO (see Example XII, below) using standard techniques commonly known to those of ordinary skill in the art. Further, the present invention makes possible the characterization of the autoimmune TCR specific to the TPO using methods described in, for example, Burns, F., et al., J.

5           Exp. Med. 169: 27 (1989). If the autoimmune T cells can be  
          eliminated or prevented from reacting with the TPO, the  
          effects of thyroiditis may be greatly alleviated. T cells  
          that will accomplish this objective may be generated which are  
          specific for the autoimmune TCR for TPO using methods  
          described in, for example, Acha-Orbea, H., et al., Ann. Rev.  
Immunol. 7: 371 (1989).

10           The manner and method of carrying out the present  
          invention may be more fully understood by those of skill by  
          reference to the following examples, which examples are not  
          intended in any manner to limit the scope of the present  
          invention or of the claims directed thereto.

15

EXAMPLE IConstruction of a Human Graves' Thyroid cDNA Library

20           A thyroid cDNA library was constructed to maximize the  
          inclusion of full-length cDNA in the coding orientation.  
          Hyperplastic thyroid tissue was obtained from a patient  
          undergoing thyroidectomy for Graves' disease. mRNA was  
          isolated according to the method of Han et al. (Han, J.H., et  
al., Biochem. 26:1617-1625 (1987)). Double-stranded cDNA was  
          synthesized from 15 µg mRNA as described by Gubler and Hoffman  
25           (Gubler, U., et al., Gene 2:263-269 (1983)). Not I and Xba I  
          linker-primers/adaptors were incorporated into the cDNA to  
          create those restriction sites at the 5' and 3' ends,  
          respectively, of the cDNA (Han, J.H., et al., Biochem.  
          26:1617-1625 (1987)). The cDNA was size-selected (> 1 kb) by  
          agarose gel (Seaplaque, FMC, Rockland, ME) electrophoresis,  
          digested with Not I and Xba I, ligated into Not I- and Xba I-  
          cut bacteriophage lambda-Zap using T4 DNA ligase, and packaged  
30           (Giga-Pak Gold, Stratagene, San Diego, CA). The resulting

phage library contained a total of  $2 \times 10^4$  recombinant clones before amplification.

EXAMPLE II

5

Screening for Full-length Human TPO cDNA

The amplified cDNA library was plated at a density of  $4 \times 10^4$  pfu per 150 mm diameter dish and probed using the insert from a partial human TPO cDNA clone (clone 19). Two positive 10 bacteriophage clones were isolated. A Bluescript phagemid containing the human TPO cDNA insert was generated from one of these clones using the helper phage R408, according to the Stratagene protocol. The resulting recombinant Bluescript 15 plasmid (pHTPO-BS) contained bases 5-3060 of human thyroid peroxidase cDNA, including the start of translation and the poly-A tail. DNA sequence was determined from this double-stranded plasmid using the Sequenase kit and protocol (United States Biochemical, Cleveland, OH). Sequence within the cDNA was confirmed to be identical to human TPO cDNA at the 5' and 20 3' ends and in the regions adjacent to 10 oligonucleotide primers distributed throughout the cDNA (Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861 (1987)).

EXAMPLE III

25

Construction of pHTPO-ECE

The mammalian cell expression vector pECE (Ellis, L., et al., Cell 45:721-732 (1986)) was obtained from Dr. William Rutter (U.C.S.F.). Human TPO cDNA was cloned into the 30 multiple cloning site of this vector as described in Figure 1. Enzyme reactions and DNA manipulations were performed as described in Maniatis et al. (Maniatis, T., et al., Molecular Biology: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)).

EXAMPLE IVTransfection of Chinese Hamster Ovary Cells with pHTPO-ECE

5        Chinese hamster ovary cell line CHO-K1 was maintained in Hams' F-12 medium supplemented with 10% fetal bovine serum, penicillin (125 units/ml), streptomycin (100 µg/ml) and amphotericin-B (2.5 µg/ml). Transfection and selection with G-418 (GIBCO, Grand Island, NY) was carried out by the method  
10      of Chen and Okayama (Chen, C., et al., Mol. Cell. Biol., 7:2745-2752 (1987)). 20 µg pHTPO-ECE plus 2 µg pSV2-neo (28)  
15      (from Dr. John Baxter, U.C.S.F.) were used for the transfection. Control transfections with 20 µg pECE plus 2 µg pSV2-neo, and 20 µg pSV2-neo alone, were performed concurrently.

EXAMPLE VRNA Extraction and Northern Blot Analysis

20       Total cellular RNA was extracted by the method of Chomczynski and Sacchi (Chomczynski, P., et al., Anal. Biochem. 162:156-159 (1987)). 15 µg of RNA was electrophoresed in formaldehyde gels as described by Maniatis et al. (Maniatis, T., et al., Molecular Biology; A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)). RNA was blotted onto a Zeta-Probe membrane (BioRad, Richmond, CA) and probed with a 0.56 kb human TPO cDNA probe (clone 31 insert), labeled to a specific activity of  $4 \times 10^9$  cpm/µg DNA using the Multi-Prime labelling kit from Amersham (Arlington Heights, IL).

EXAMPLE VIWestern Blot Analysis

Transfected CHO cells were extracted to obtain soluble protein. Five 100 mm diameter dishes were washed 3 times with calcium-magnesium free phosphate-buffered saline (PBS). After aspiration, 5 ml of 0.5% Triton X-100 in the same buffer, supplemented with 10 µg/ml leupeptin, 0.5 mg/ml bacitracin and 2 mM phenylmethylsulfonyl fluoride (all from Sigma, St. Louis, MO), were added to the first dish. This initial cell solution was scraped and transferred successively to the other 4 dishes of cells. The cell solution was then tumbled for 1 hour at 4°C. After centrifugation for 3 minutes at 10,000 x g, the supernatant was saved and stored at -20°C until use.

Protein content was determined (Bradford, M.M., Anal. Biochem. 72:248-254 (1976)) and 50 µg protein/lane electrophoresed on a 7.5% polyacrylamide SDS gel (Laemmli, U.K., Nature 227:680-689 (1970)). Proteins were electrotransferred (30 V x 5 hours, or 250 mA overnight) to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in an electroblotting apparatus (Hoeffer, San Francisco, CA) containing 25 mM Tris, 192 mM glycine, 20% methanol. In later experiments, transfer was accomplished using a Polyblot semi-dry electrotransfer system (American Biometric, Hayward, CA), according to the directions of the manufacturer. Membranes were rinsed once in TBS (0.1 M Tris, pH 8.0, 0.15 M NaCl), then for 30-60 minutes at room temperature in TBS containing 0.5 % Tween 20 (Sigma, St. Louis, MO). After 3 further rinses with TBS-Tween, the blots were probed as described by Young and Davis (Young, R.A., et al., In Genetic Engineering: Principles and Methods, Plenum Publishing Corp., 1:29-41 (1985)) using a 1:250 dilution of a mouse mAb against the thyroid microsomal antigen (Portmann, L., et al., J. Clin. Invest. 81:1217-1224 (1988)), followed

-44-

by a 1:250 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Sigma, St. Louis, MO).

In other experiments, CHO-HTPO12b cell extracts were probed using a panel of polyclonal Hashimoto's thyroiditis sera, provided by Dr. S. M. McLachlan, University of Wales, Cardiff. Antimicrosomal antibody titers had previously been determined by enzyme-linked immunosorbant assay (ELISA) in the presence of excess thyroglobulin (Jansson, R., *et al.*, *Clin. Exp. Immunol.*, **63**:80-86 (1986)). Multiple Hashimoto's thyroiditis sera were applied to a single filter overnight at 4°C using a Miniblotter 45 manifold (Immunetics, Cambridge, Mass.). Membranes were then processed as described above, except that alkaline phosphatase-conjugated goat anti-human IgG, Fc fragment specific (Cappel, Organon Teknica Corp., West Chester, PA) was used as the second antibody with nitroblue tetrazolium (0.3 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (0.15 mg/ml) in 100mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl<sub>2</sub>.

20

#### EXAMPLE VII

##### Fluorescence-activated Cell Sorter (FACS) Analysis

25

CHO-HTPO12b cells were processed as described by Ellis *et al.* (Ellis, L., *et al.*, *Cell* **45**:721-732 (1986)). In brief, cells from a 100 mm diameter dish were detached by mild trypsinization, and the cells rinsed and pelleted (5 minutes at 100 x g, 4°C) in Ham's F12 medium, 10% fetal calf serum (see above). The cells were resuspended in 0.2 ml of phosphate-buffered saline (PBS), 10mM Hepes, pH 7.4, 0.05% Na azide (buffer A). Serum to be tested (2ul) was added for 30 minutes at 4°C, followed by two rinses in buffer A with 2% fetal calf serum and resuspension in 0.2 ml of the same solution. 25 ul of goat anti-human IgG, Fc specific, affinity-purified, R-Phycoerythrin-labeled (Caltag, South San

Francisco, CA) were added for another 30 minutes at 4°C. After 3 washes in buffer A, the cells were analyzed on a fluorescence-activated cell sorter.

5

**EXAMPLE VIII**  
**Assay of Human TPO Enzymatic Activity**

Human TPO activity was assayed following extraction from cell microsomes with trypsin and deoxycholate as previously 10 described (Magnusson, R.P., *et al.*, *Endocrinol.* **116**:1493-1500 (1985)). In later experiments, a more rapid method was used. Cells were suspended with a rubber scraper in 1.5 ml calcium-magnesium free Dulbecco's phosphate-buffered saline and protein determined on a 5  $\mu$ l aliquot. The cells were then 15 pelleted in a microcentrifuge for 2 minutes. Cold 0.1% deoxycholate (0.2 ml/mg cellular protein) was added for 10 minutes. The extract was microcentrifuged for 5 minutes and the supernatant removed for assay. One guaiacol unit is defined as a  $\Delta$ 470 of 1.0 per minute which is equivalent to 20 150 nmols guaiacol oxidized per minute (Chance, B., *et al.*, In *Methods in Enzymology* (Colowick, S.P., *et al.*), Academic Press, New York **2**:764-775 (1955)). One unit of iodide peroxidase is defined as a  $\Delta$ 353 of 1.0 per minute which corresponds to 43 nmols I<sub>3</sub>- formed per minute (Magnusson, 25 R.P., *et al.*, *J. Biol. Chem.* **259**:13783-13790 (1984)).

**EXAMPLE IX**  
**Primary Culture of Human Graves' Disease Thyroid Cells**

30

Human Graves' disease thyroid tissue was dispersed and the cells cultured as previously described (Hinds, W.E., *et al.*, *J. Clin. Endocrinol. Metab.* **52**:1204-1210 (1981)). After 3 days in culture, fresh medium containing 12.5 mU/ml TSH was

-46-

added for an additional 3 days before the cells were harvested and extracted as described above for the western blots.

EXAMPLE X

5       Comparison of Recombinant hTPO and Microsomal Antigen as Sources of Antigen for ELISAs for Anti-MSA/Anti-TPO Antibodies

10      Sera from 51 individuals were provided by Dr. S. M. McLachlan (University of Wales College of Medicine, Cardiff, U.K.). Forty seven of these sera were from patients with autoimmune thyroid disease, selected to represent a balanced spectrum of anti-MSA titers from low to very high. Four sera were from normal individuals. Anti-MSA and anti-TGA antibodies were measured by the method of Schardt et al. (Schardt, C.W., et al., J. Immunol. Methods **55**:155-168 (1982)) and the method of Endo et al. (Endo, Y., et al., Clin. Chim. Acta **103**:67-77 (1980)), as modified by McLachlan et al. (McLachlan, S.M., et al., Immunol. Letters **4**:27-33 (1982)), respectively. For the anti-MSA assay, human thyroid microsomes were prepared from frozen Graves' thyroid tissue obtained at operation for the treatment of this disease (Schardt, C.W., et al., J. Immunol. Methods **55**:155-168 (1982)). In order to avoid cross-reactivity of patients' sera with any thyroglobulin remaining in the microsomal preparation, sera were pre-adsorbed in buffer containing 100 µg/ml ( $1.5 \times 10^{-6}$  M) thyroglobulin (obtained from the same tissue) at 4°C overnight and thereafter at room temperature for 2 hours before assay (Schardt, C.W., et al., J. Immunol. Methods **55**:155-168 (1982)).

20      The generation of Chinese hamster ovary (CHO) cells (clone CHO-HTPO 12b) expressing enzymatically-active human TPO has been described above. These cells had been transfected with the recombinant plasmid pHTPO-ECE, constructed by the insertion of a full-length human TPO cDNA into the expression vector pECE. CHO-HTPO 12b and CHO-K1 (control, non-

transfected) cells were grown in Ham's F-12 medium supplemented with 100 g/L fetal bovine serum (FBS), penicillin (125 units/ml), gentamicin (48 µg/ml) and amphotericin-B (2.5 µg/ml). Cells were grown to confluence in 100 mm dishes, the  
5 cells were rinsed three times with Dulbecco's calcium-magnesium free, phosphate-buffered saline (PBS), and then scraped into a solution containing 10 mM Tris, pH 7.4, 0.25 M sucrose, 2 mM phenylmethyl sulfonyl fluoride, 10 µg/ml leupeptin, 0.5 mg/ml bacitracin (Buffer A). Cells were  
10 homogenized for 20 seconds with a Polytron, centrifuged for 15 minutes at 10,000 x g, 4°C, and the supernatant then centrifuged for 1 hour at 100,000 x g, 4°C. The microsomal pellet was resuspended in 0.5 ml of Buffer A, homogenized in a Dounce homogenizer, and then frozen at -80°C until use.  
15 Protein content was determined by the method of Bradford (Bradford, M.M., Anal. Biochem. 72:248-254 (1976)). Yield of microsomal protein was approximately 100-200 µg per 100 mm dish of confluent cells.

Sera to be tested were stored in aliquots at -80°C before  
20 use. The assay procedure was that of Schardt et al. (Schardt, C.W., et al., J. Immunol. Methods 55:155-168 (1982)), with slight modifications. Multiwell micro-ELISA plates (Dynatech Labs, Chantilly, VA) were coated (overnight at 4°C) with 4 µg CHO-HTPO 12b or CHO-K1 microsomal protein per well in coating  
25 buffer (0.05 M sodium bicarbonate, pH 9.3, 0.02% sodium azide). The wells were then rinsed twice in 0.2 M Tris, pH 7.4, 0.15 M NaCl (Tris buffer), once in 0.2 M Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween 20 (Tris-Tween buffer), and once in Tris buffer. 100 ul of PBS, 50 g/L bovine serum albumin (BSA)  
30 (Sigma, St. Louis, MO) were added to each well and incubated for 20 minutes at room temperature. After aspiration, the wells were washed twice in Tris buffer, once in Tris-Tween buffer, and once in Tris buffer.

-48-

Serum samples were diluted 1/100, 1/1000 or 1/10,000 in PBS, 5 g/L BSA. 100  $\mu$ l of the diluted serum sample were added per well in duplicate and incubated for 1 hour at 37°C. The wells were then washed three times with PBS. 100  $\mu$ l of 5 peroxidase-conjugated, affinity-purified, goat anti-human IgG, Fc fragment specific antibody (Cappel, Organon Teknika Corp., West Chester, PA.), diluted 1/500 in PBS, 250 g/L FBS, were added to each well and incubated for 1 hour at 37°C. The wells were then washed four times with Tris-Tween buffer. 10 100  $\mu$ l of substrate solution (12 ml of 0.23 M citrate, 0.26 M sodium phosphate, pH 5.0 solution + 12  $\mu$ L 30% H<sub>2</sub>O<sub>2</sub> + 4.2 mg ortho-phenylenediamine) were added to each well and incubated for 30 minutes at room temperature. The reaction was stopped by adding 100  $\mu$ l of 20% sulfuric acid to each well. ELISA 15 values (OD 490 nm) were measured in a micro-ELISA reader and normalized (blanked) to a well lacking antigen.

#### EXAMPLE XI

##### Oligonucleotide-Directed Mutagenesis of Human TPO cDNA

20 A. METHODS

The non-coding strand of human TPO cDNA, in the phagemid Bluescript (Stratagene, San Diego, CA), was used as a template for oligonucleotide-directed mutagenesis. A 52 bp mutagenic primer

25 (5'-AGGCTCCCTCGGGTGACTTGAATTCCATGTAGCTGGCTGCTTGATCG-3'), synthesized by the Molecular Genetics Core Facility, San Francisco Veterans' Administration Medical Center, was designed to generate two stop codons directly upstream of the putative membrane-spanning region of the protein. Thus, TGA 30 and TAG codons were created at 2629-2631 bp and 2641-2643 bp in human TPO cDNA (Magnusson, R.P., *et al.*, *Mol. Endocrinol.* 1:856-861 (1987)), respectively. For convenient screening of mutants, an Eco RI restriction site (GAATTC, at 2630-2635 bp) was created together with the first (TGA) stop codon. The

mutagenesis procedure was performed according to the protocol of the manufacturer (Muta-gene phagemid in vitro mutagenesis kit, Biorad, Richmond, CA) to generate the plasmid pHTPO(M1)-BS.

5        After confirmation of the mutation by nucleotide sequencing (Sanger, F., et al., Proc. Natl. Acad. Sci. USA 74:5463-5467 (1977)) (Fig. 13), the cDNA was excised by digestion of pHTPO(M1)-BS with Not I, the ends blunted with the Klenow fragment of DNA Polymerase I, and the cDNA  
10      liberated by digestion with Xba I. The mutated cDNA (3.05 Kb) was substituted for wild-type human TPO cDNA in the plasmid pSV2-DHFR-ECE-HTPO, to generate pHTPO(M1)-ECE-SV2-DHFR. This plasmid contains components of the expression vectors pECE (Ellis, L., et al., Cell 45:721-732 (1986)) and pSV2-dhfr (Lee, F., et al., Nature 294:228-232 (1981)), provided by Dr. William Rutter (University of California, San Francisco) and Dr. Gordon Ringold (Syntex, Palo Alto), respectively. In brief, pSV2-DHFR-ECE-HTPO was digested with Sal I, the ends blunted with the Klenow fragment of DNA polymerase I, and the hTPO cDNA released by digestion with Xba I. The remaining vector (pSV2-DHFR-ECE) was treated with bacterial alkaline phosphatase, gel purified, and recovered in SeaPlaque agarose (FMC BioProducts, Rockland ME). Mutated hTPO cDNA, also recovered in SeaPlaque agarose, was ligated into this vector.  
15  
20  
25      Enzymes.    Restriction enzymes, T4 DNA ligase and DNA polymerase I, Klenow fragment were obtained alternatively from Bethesda Research Laboratories (Gaithersburg, MD), New England Biolabs (Beverly, MA) or Boehringer-Mannheim (Indianapolis, IN).

30

#### B. RESULTS AND DISCUSSION

Because multiple screenings of previously constructed human thyroid cDNA library in lambda gt11 (Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861 (1987)) only yielded

fragments of TPO cDNA, a new thyroid cDNA library in lambda-Zap was constructed as described herein. The plasmid pHTPO-BS containing full-length human TPO cDNA was obtained from this library. pHTPO-ECE was constructed from pHTPO-BS and the mammalian expression vector pECE (Ellis, L., *et al.*, *Cell* 45:721-732 (1986)) according to the strategy shown in Figure 1, and was used for subsequent cell transfections.

Chinese hamster ovary cells were co-transfected with pHTPO-ECE and pSV2-neo, and 12 clones were tested for the presence of TPO mRNA by northern blot analysis. Total cellular RNA (15 µg/lane) from four pHTPO-ECE transfected cell lines (CHO-HTPO4, CHO-HTPO12, CHO-HTPO14 and CHO-HTPO17), and one control pSV2-neo-transfected cell line (CHO-pSV2-neo), was subjected to northern blot analysis using a human TPO cDNA probe, as described herein. For comparison, 1 µg of poly A+ mRNA prepared from a human thyroid gland from a patient with Graves' disease was used. 28S and 18S ribosomal RNA markers, and an RNA molecular weight ladder (B.R.L., Gaithersburg, MD) were employed for molecular weight determination.

Four of these clones, as well as one of four control (pSV2-neo alone) clones, revealed a 3.3 kb mRNA band in the pHTPO-ECE-transfected clones. The size of the human TPO mRNA in the transfected CHO cells is slightly larger than that in the Graves' thyroid cells (3.1 kb), presumably because of the additional SV40 poly-A coding region at the 3' end of human TPO cDNA in the pHTPO-ECE plasmid (see Figure 1).

Western blot analysis (under reducing conditions) of proteins extracted from TPO-transfected CHO cells, using a mouse monoclonal anti-human thyroid microsomal antibody (Portmann, L., *et al.*, *J. Clin. Invest.* 81:1217-1224 (1988)), revealed an immunoreactive protein of 105-110 kD, as expected for human thyroid peroxidase (Czarnocka, B., *FEBS Letters* 109:147-152 (1985); Ohtaki, S., *et al.*, *J. Clin. Endocrinol.*

Metab. 63:570-576 (1986)). Briefly, 50 µg of membrane protein or 30 µg of deoxycholate (DOC)-extracted protein from pHTPO-ECE-transfected cell lines (CHO-HTPO4, CHO-HTPO12, CHO-HTPO14, CHO-HTPO17), from a control cell line co-transfected with pECE 5 and pSV2-neo, and from another control cell line transfected with pSV2-neo alone, were subjected to SDS polyacrylamide gel electrophoresis under reducing conditions. The proteins were 10 electrotransferred to nitrocellulose membranes and then probed, as described herein, with a mouse mAb against the thyroid microsomal antigen (Portmann, L., *et al.*, J. Clin. Invest. 81:1217-1224 (1988)).

Strong TPO enzymatic activity was evident in clone CHO-HTPO12, and in subclones CHO-TPO12b and CHO-TPO12g, obtained by limiting dilution (Table I). Less enzymatic activity was 15 detected in the other clones. TPO activity in the CHO-TPO12 clones was approximately the same as TPO activity in TSH-stimulated Graves' thyroid cells in monolayer culture (Table I).

In order to determine whether, as with native TPO in 20 thyroid cells, the recombinant, human TPO was expressed on the surface of the CHO cells transfected with this gene, CHO-HTPO12b cells were subjected to FACS analysis (Figure 2). Incubation of these cells with high-titer MSA Hashimoto's serum (ELISA value of 1.772; normal < 0.2) (Jansson, R., *et 25 al.*, Clin. Exp. Immunol. 63:80-86 (1986)) yielded approximately 100-fold greater fluorescence than when these cells were incubated with control serum (Figure 2). Similar results were obtained with three different Hashimoto's sera. The size of both the control and Hashimoto's serum-incubated 30 cells was the same (Figures 3E and 3F), excluding the possibility that differences in cell size were, in part, responsible for the differences in signal.

A series of western blot studies was then performed with protein from CHO-TPO12b cells using a panel of Hashimoto's

sera with known antimicrosomal antibody levels as determined by ELISA (Jansson, R., et al., Clin. Exp. Immunol. **63**:80-86 (1986)). Under non-reducing conditions, all 29 Hashimoto's sera tested, unlike three normal sera, reacted with a major, 5 broad protein band of approximately 200 kD as well as with a fainter doublet of about 110 kD. In aggregate, in studies performed under non-reducing conditions, a total of 36 Hashimoto's sera tested, but not the six control sera, reacted with these bands. The interexperimental variability in the 10 intensity of these bands, however, as well as methodological limitations in analyzing many samples simultaneously, precluded comparison of results of all samples tested.

Nevertheless, it was apparent that, within a single large experiment, the strongest signals were seen with sera containing the highest antimicrosomal antibody ELISA values. Some sera also recognized protein bands other than those expected for TPO. These bands represented wild-type CHO 15 antigens (presented below). One apparent TPO-specific signal of 110 kD also was a non-specific wild-type CHO signal. This 20 is discussed in more detail below.

Comparison of the recombinant TPO signals on western blots performed under reducing and non-reducing conditions (using  $\beta$ -mercaptoethanol) revealed the following with reduction: (a) loss of the 200 kD broad band; (b) alteration 25 of the 110 kD signal so that it no longer clearly represents a doublet; and (c) lessening of the specific signals so that some of the weaker sera become negative. A non-immune serum described above that reacted with a band of approximately 110 kD represents a wild-type CHO protein, and not TPO.

The specificity of the 200 kD and 110 kD bands discussed above was demonstrated in two separate experiments utilizing wild-type, non-TPO-transfected, CHO cells. In the first experiment, selected, potent Hashimoto's sera tested under the 30 most favorable (i.e., non-reducing) conditions failed to react

with protein bands of 200 kD or 110 kD. The second experiment indicated that the non-immune serum previously shown to react with a band of 110 kD is a false-positive. This signal in wild-type CHO cells is strong despite the use of unfavorable 5 (i.e., reducing) conditions.

To assess the sensitivity of detection of the specific signal, western blot analyses were performed with serial dilutions of two Hashimoto's sera. The amount of TPO generated in CHO-TPO12b cells was sufficient to be detected 10 even when these Hashimoto's sera were diluted greater than 3000-fold.

Human TPO contains 5 potential glycosylation sites. It was therefore examined whether carbohydrate moieties are important in the conformation of the epitope(s) in the human 15 TPO antigen(s) recognized by Hashimoto's sera. Western blot analyses were performed on proteins extracted from CHO-TPO12b cells pre-cultured for 20 hours in 0.5 µg/ml tunicamycin, an inhibitor of protein glycosylation. This length of time was chosen because it was the longest tolerated without evidence 20 of significant toxicity (i.e., cell loss). Tunicamycin treatment had no apparent effect on antigen recognition, suggesting that carbohydrate moieties may not be important components of the microsomal antigen epitope(s). In a control experiment, tunicamycin treatment under similar conditions 25 decreased radiolabeled D-glucosamine incorporation into proteins by  $56.3 \pm 4.8\%$  (mean  $\pm$  S.D.; n=3).

An ELISA carried out using antibodies directed against the microsomal antigen (MSA) was compared with an ELISA performed with antibodies directed against the recombinant 30 human TPO of the present invention (Figure 3). Very good correlation (0.8385249) was observed. In fact, the anti-MSA based ELISA resulted in false positives (indicated as "outliers" in Figure 3), which were not observed in the ELISA based upon the anti-recombinant human TPO antibody.

These false positives are likely to result from non-specific reactions of antithyroglobulin antibodies with the microsomes, and were not included in the linear regression calculation for Figure 3. Support for this conclusion is 5 found in Figure 4, which shows a linear regression analysis analogous to that shown in Figure 3, but at a much greater (1/1000) dilution. It can be seen from Figure 4 that the increased dilution factor has substantially eliminated the outlying data points seen at the lower dilution, and that the 10 correlation (0.9060773) is significantly greater. This result strongly suggests that the lowered specificity of the anti-MSA based ELISA is, indeed, a function of antigen contamination. Such problems, which lower assay specificity, might be addressed by the use of non-recombinant, affinity-purified 15 TPO. However, generation of truly pure, affinity-purified natural TPO has proven to be very difficult, if not impossible, to achieve. These problems are avoided by use of the recombinant human TPO antigen of the present invention.

In order to further examine its specificity, recombinant 20 human TPO was compared with Graves' thyroid microsomes as a source of antigen in an ELISA procedure. The recombinant hTPO was present in microsomes prepared from a non-thyroidal, non-human eukaryotic cell line which cannot, therefore, contain thyroid-specific antigens other than hTPO. Nevertheless, 25 because sera from patients with autoimmune thyroiditis contain antibodies against numerous antigens, some of which may be present in Chinese hamster ovary (CHO) cells (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)), each serum sample was also assayed against microsomes prepared from 30 control, non-transfected CHO cells.

In comparing the 51 sera at a standard (1/100) dilution in both the recombinant hTPO and the thyroid microsomal assay, a moderately good correlation was observed ( $r=0.668$ ;  $p<0.001$ ) (Fig. 10A). Clearly, however, there were some widely

discrepant values. In particular, two sera (sera #11 and 27, Fig. 10A, large circle and square, respectively) that were very potent in the anti-MSA assay gave values in the anti-hTPO antibody assay similar to the four normal sera (Fig. 10A, four data points within rectangle near the origin). A number of other sera, primarily in the high range of activity, also gave significantly higher values with the thyroid microsomal preparation than with recombinant hTPO (Fig. 10A). At the same serum dilution, a much lower correlation was observed between the values obtained with thyroglobulin and recombinant hTPO as antigen ( $r=0.315$ ;  $p<0.05$ ).

In an autoimmune serum containing antibodies against multiple antigens, the different antibodies are likely to have varying affinities for their respective antigens. Serial dilutions of sera will yield different profiles of ELISA values based on the affinity of each antibody-antigen interaction. If hTPO is the primary autoantigen in the thyroid microsomal preparation, the same serum dilution curve should therefore be observed in assays using thyroid microsomes and recombinant hTPO. In support of this hypothesis, at serum dilutions of 1/1000 or 1/10,000, the correlation in ELISA values between thyroid microsomes and human TPO was much greater ( $r=0.906$  and 0.902, respectively;  $p<0.001$ ) (Figs. 10B and 10C). Dramatically, the two sera that were strongly positive with the thyroid microsomal but not with the recombinant hTPO antigen (Fig. 10A) were no longer significantly discrepant between the two assays (Fig. 10B and 10C). The dilution curves for these two sera were quite different in the anti-MSA and anti-hTPO antibody assays (Fig. 11A and 11B), confirming that these sera were reacting with low affinity to an antigen other than hTPO. These two sera were also distinguished by their surprisingly high levels of anti-thyroglobulin antibody. In contrast, other sera with similar anti-MSA levels (at 1/100 serum dilution) yield normal

dilution curves in both assays (sera #12 and 28, Fig. 11A and 11B).

The anti-hTPO antibody ELISA data were also expressed as the difference between values obtained using the CHO-hTPO microsomes and the CHO-K1 microsomes as antigen, to correct 5 for possible interference by anti-CHO cell antibodies (Kaufman, K.D., *et al.*, *J. Clin. Invest.*, **84**:394-403 (1989)). No significant change was found in the correlation between the 10 thyroid microsomal and the recombinant hTPO assays using these revised data at each of the three serum dilutions. Anti-CHO-K1 antibody ELISA values for the 47 sera of patients with autoimmune thyroid disease tested, at standard (1/100) dilution, were  $0.164 \pm 0.066$  SD (mean  $\pm$  SD).

The precision of the anti-hTPO antibody ELISA was 15 assessed using three sera chosen to represent a spectrum of autoantibody potency. Intra-assay variability (10 iterations for each serum) at standard (1/100) serum dilution, expressed as mean  $\pm$  SD (Fig. 12), was  $0.346 \pm 0.18$  (low-potency serum),  $0.599 \pm 0.44$  (medium-potency serum), and  $0.923 \pm 0.94$  (high-potency serum). The intra-assay coefficients of variation 20 (CV) for these sera were 5.12%, 7.39%, and 10.2%, respectively. The inter-assay CV's (7 iterations for each serum) were 5.36%, 7.63%, and 7.29%, respectively.

In another aspect of the present invention, it has 25 surprisingly been discovered that CHO cell expression of human TPO can be significantly increased by employing a different plasmid. A dihydrofolate reductase (DHFR)-TPO construct has been made in which both genes (DHFR and TPO) are driven by the SV40 promoter (Figure 4). Screening of CHO cells transfected 30 with these constructs has produced two plasmids, designated pHTPO-DHFR-2B and pHTPO-DHFR-4C, which presently express three-fold more antigen than that achieved using the pECE-hTPO plasmid.

The relative TPO activities observed in CHO cells transfected with pECE-HTPO, pHHTPO-DHFR-2B and pHHTPO-DHFR-4C are shown plotted against methotrexate concentration in Figure 5. Further, one particular subclone, designated pHDFR-TPO-4C-MTX, has been found to express relatively greater amounts of TPO than any other construct so far isolated, and, in this regard, comprises the best mode presently contemplated of expressing human TPO in CHO cells. The plasmid pHDFR-TPO-4C-MTX was deposited at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852 on October 3, 1989, with accession number CRL 10250.

Figure 5 shows that, with increasing methotrexate concentrations, a plateau is reached for CHO expression of TPO by the pHHTPO-DHFR-2B and pHHTPO-DHFR-4C plasmids. While not intending to be bound by any particular theory, one possible explanation for this observation is that the expressed full length TPO gene is toxic to the host CHO cells, resulting in selection for DHFR, but against TPO, at higher methotrexate concentrations. The result of such selection might be that DHFR is amplified while TPO is deleted.

Since the full length TPO gene is membrane-associated, the present inventor hypothesized that it may be possible to increase TPO production in CHO cells if the expressed protein could somehow be dissociated from the membrane. Accordingly, experiments have been undertaken to generate a secretable form of human TPO, by identifying and eliminating the wild-type transmembrane sequence from the gene.

Premature termination in the synthesis of hTPO was hypothesized to reduce the size of the hTPO-M1 protein from 933 to 848 amino acids. An original full length human TPO cDNA clone in Bluescript (pHTPO-BS) was submitted to site-directed mutagenesis to produce plasmid pHHTPO(M1)-BS. A single-stranded DNA template was generated, and the indicated 52-mer oligonucleotide probe used for mutagenesis. The

mutations incorporated two stop codons, as well as an EcoR1 site for confirmation, in the region immediately upstream from the transmembrane region of the human TPO gene (Figure 6). The entire full length human TPO gene sequence is shown for comparison in Figure 7.

As a consequence of the mutation, a "truncated" human TPO protein is expressed which is secreted by the host cell rather than bound to its membrane. The mutated hTPO gene was excised from pHTPO(M1)-BS using Not I (blunted with Klenow polymerase) and Xba I, and was inserted into the corresponding sites of pECE-SV2-DHFR, to produce the expression plasmid pHTPO(M1)-ECE-SV2-DHFR (Figure 8). CHO cells transfected with this plasmid appear to produce a truncated human TPO protein, which is believed to retain the antigenic properties of the full length protein, and which, accordingly, comprises another embodiment of the present invention. Construction of the plasmid pHTPO(M1)-ECE-SV2-DHFR is summarized in Figure 9.

After stable transfection of CHO cells with the plasmid pHTPO(M1)-ECE-SV2-DHFR containing the mutated hTPO cDNA, individual colonies of cells (CHO-TPO-M1) were studied for the expression of TPO (Fig. 14).

Because the kinetics of potentially-secreted hTPO-M1 protein were unknown, the expression of this protein was initially screened for in CHO cell lysates, since particulate TPO would be expected to be detectable even if the protein were, in large part, secreted. Randomly selected CHO-TPO-M1 clones showed evidence of variable cellular TPO expression (Fig. 14). A doublet of approximately 105-101 kD was specifically immunoprecipitated from lysates of these clones by serum from a patient with Hashimoto's thyroiditis. In CHO cells transfected with wild-type hTPO cDNA, Hashimoto's serum immunoprecipitated a doublet of larger size, 112-105 kD, and neither doublet was detected in non-transfected CHO cells (Fig. 14A), as previously observed (Kaufman, K.D., et al., J.

5           Clin. Invest. 84:394-403 (1989)). Immunoreactive TPO was absent from the cell surface of the CHO-TPO-M1 cells, as demonstrated by the lack of immunofluorescence when these cells were pre-incubated with Hashimoto's thyroiditis serum  
10           and fluorescently-tagged goat anti-human IgG antibody, unlike CHO cells transfected with wild-type hTPO (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)).

15           In order to determine whether mutated hTPO-M1 is a secreted protein, the biosynthesis and processing of both hTPO-M1 and wild-type hTPO was examined in pulse-chase experiments. First, clone CHO-TPO-M1-K, with the highest expression of truncated TPO (Fig. 14A), was subcloned by limiting dilution, and one cell line (CHO-TPO-M1-K1) was selected for further studies (Fig. 14B). Over a 24 hour chase period, radiolabeled hTPO-M1 protein was secreted by cells into the culture medium and detected by immunoprecipitation with Hashimoto's serum (Fig. 15). This secreted protein was present in the culture medium after 4 hours of chase, with levels accumulating progressively over a 24 hour period.  
20           Interestingly, the secreted, immunoprecipitable hTPO-M1 protein appeared as a single band of lesser electrophoretic mobility on the polyacrylamide gel, as compared with its cell-associated form. In contrast, CHO cells expressing wild-type hTPO secreted no detectable immunoprecipitable material into the culture medium. The cell-associated hTPO and hTPO-M1 proteins were similarly stable, with their radiolabeled immunoprecipitates increasing between 0 and 4 hours of chase. Amounts of radiolabeled, immunoprecipitable wild-type hTPO protein at 24 hours of chase were similar to baseline (0 hours). The observed decrease in signal in CHO-TPO-M1-K1 cell lysates from 4 to 24 hours is paralleled by an increase in signal in the medium of these cells, supporting the concept of a secreted protein, which, accordingly, comprises another embodiment of the present invention.  
25  
30

-60-

In order to prove that the immunoprecipitable material released into the culture media by CHO-TPO-M1-K1 cells was, indeed, TPO, conditioned media were tested for TPO enzymatic activity. TPO activity (1.0 guaiacol U/10 ml medium) was clearly present in the culture medium from the CHO cells expressing the mutated form of hTPO (Fig. 16). In contrast, there was no detectable enzymatic activity in conditioned media from CHO cells expressing wild-type hTPO (Fig. 16), despite strong TPO activity present in lysates of these cells, as previously described (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)).

Table I

Thyroid Peroxidase (TPO) Activity in CHO-TPO12 Cells  
and in TSH-Stimulated Graves' Disease  
Human Thyroid Cell Primary Cultures

	Cell Type	Guaiacol Peroxidase (units/mg protein)	Iodide Peroxidase (units/mg protein)
20	CHO-pECE (control)	0	0
25	CHO-pSV2-neo (control)	0	0
	Human thyroid cells	4.7	3.0
		4.6	3.4
30	CHO-HTPO12	3.6	nd
	CHO-HTPO12b	4.0	3.1
	CHO-HTPO12g	3.1	1.9
35	Summary of data from multiple determinations of guaiacol and iodide TPO activity measured in deoxycholate extracts, prepared from 100 mm diameter dishes of the indicated cells. Graves' disease-affected human thyroid cells were cultured for 3 days in 12.5 mU/ml human TSH.		
40	nd - not done		

EXAMPLE XIITPO Specific T Cells Infiltrate Thyroid in Graves' Disease

5        Taking advantage of the availability of recombinant TPO, the occurrence of in vivo selection for T cells specific for this autoantigen in the intrathyroidal population has been examined.

10      A. METHODS

Infiltrating mononuclear cells were extracted from the thyroidectomy specimen of a 26 year old female (CX81:HLA-A1, 2; B8, 37; DR3; DRw52; DQw2) with persistently relapsing Graves' disease and a high titer of antithyroid microsomal antibodies (1:640) by enzyme digestion followed by overnight incubation and separation of the non-adherent cells as previously described (Londei, M. *et al.*, *Science* 228:85-89 (1985)). The activated cells were selectively expanded by growth in recombinant IL-2 (Ajinomoto - 20 ng/ml) and 10% human serum in RPMI-1640 (Gibco) for one week. Cells were further expanded, nonspecifically with the addition of irradiated autologous peripheral blood lymphocytes as feeder cells, OKT3 monoclonal antibody (30 ng/ml) and IL-2 for two weeks prior to cloning at limited dilution (0.5 cells/well) with OKT3/IL-2 and DR-matched antigen presenting cells (APC). Further expansion and maintenance of all clones was by 1-2 weekly restimulations with OKT3/IL-2 and HLA unmatched irradiated feeder cells. Cells were assayed at the end of the feeding cycle and a minimum of 5 days after their last exposure to IL-2.

Proliferation assays were performed over 3 days in triplicate microtiter wells. Irradiated autologous PBL (2 - 5 x 10<sup>4</sup>) were added to 10<sup>4</sup> clone T cells in 200 µl of 10% human serum. 1 µl of neat microsome (protein concentration 5 mg/ml)

was added per well. 1  $\mu$ Ci of [ $^3$ H]thymidine was added for the final 6 hours of the assay prior to harvesting onto glass fiber filters and scintillation counting.

5 Peripheral blood mononuclear cells purified by sucrose gradient centrifugation (Lymphoprep - Nycomed) were incubated at  $10^5$  cells per well in microtiter wells containing 200  $\mu$ l 10% human serum. Control or TPO microsomes in 1-2  $\mu$ l were added per well as above. Cultures were incubated for 5-6 days and pulsed with [ $^3$ H]thymidine in the last 6-18 hours prior to 10 harvesting and scintillation counting.

Transfection of CHO cells with the complete cDNA for Human TPO cloned into the expression vector pECE and the preparation of cell microsomes from transfected and untransfected CHO cells was as described above.

15

B. RESULTS

In vivo activated thyroid infiltrating T cells were selected by growth in recombinant IL-2. The resultant population was then further expanded non-specifically by 20 stimulation with anti-ClB3 antibodies (OKT3) in combination with IL-2. Lines so derived consistently showed a marked response to autologous thyroid epithelial cells in the absence of added antigen-presenting cells (APC). For example, the following levels of T cell stimulation, measured as 25 incorporation of radiolabeled thymidine, were observed:

T cells:  $51 \pm 3$  cpm;

Thyroid epithelial cells (TEC):  $62 \pm 8$  cpm;

T cells + TEC:  $6108 \pm 1040$  cpm.

T cell clones were obtained by plating the lines at 30 limiting dilution (0.5 cells/well) followed by further expansion with IL-2 and OKT3. In this way, antigen-specific selection was avoided prior to screening of the clones.

The complete sequence of human TPO cDNA was cloned into the mammalian expression vector pECE and transfected into

Chinese Hamster Ovary (CHO) cells as described above. These transfected cells express high levels of immunoreactive and enzymatically active TPO. Microsomes prepared from transfected CHO cells were found to induce significant 5 proliferation of 5 of 24 clones derived from the intrathyroidal population (Figure 17A). These cells showed no response to untransfected CHO microsomes (Figure 17A).

In contrast, peripheral blood T cells (PBL) from the same individual, from other Graves' patients, or from normal 10 controls, responded to both transfected and untransfected preparations (Figure 17B). PBL reactivity to CHO cell derived proteins is not unexpected as similar reactivity has been described with other xenogeneic cell extracts (Van Vliet, E. et al., Europ. J. Immunol. 19:213-216 (1989)). However, it 15 demonstrates the difference in antigenic repertoire between thyroid infiltrating and peripheral blood T cells, as at no time was any response to untransfected CHO microsomes seen with thyroid-derived T cells (Figure 17A and Table IV).

-64-

TABLE II  
Sites Of NP Synthetic Peptides In Human TPO

5	1	
	933	
10	NP-1 111 ---- 131	
	NP-2 116 ---- 131	
	NP-3 187 ---- 204	
	NP-4 234 ---- 250	
15	NP-6 426 ---- 440	
	NP-7 535 ---- 551	
	NP-8 669 ---- 686	
	NP-9 693 ---- 716	
	NP-10 724 ---- 739	
	NP-13 487 ---- 504	
20		

Position of synthetic peptides used to screen T cells in human TPO sequence. Residues are numbered from the amino-terminus.

25 TABLE III  
Responses of T Cell Clone c43 to NP Peptides

30	<u>Peptide</u>	<u>Concentration (<math>\mu</math>g/<math>\mu</math>l)</u>		
		0.1	1.0	10
35	NP-1 48	72	52	
	NP-2 85	54	63	
	NP-3 59	62	62	
	NP-4 50	67	100	
	NP-6 65	60	102	
	NP-7 271	6190	16235	
40	NP-8 68	85	221	
	NP-9 69	52	80	
	NP-10 63	101	55	
	NP-13 38	69	121	

45 Responses (in counts per minute, cpm) of thyroid derived T cell clone c43 to the panel of synthetic peptides of Table II. Peptides were used at the concentrations shown. Response of c43 + autologous feeders alone was  $101 \pm 16$  cpm. S.E.M. of responses was consistently less than 15% of the mean. The response of c43 to NP-7 was confirmed in 5 subsequent experiments with similar results.

TABLE IV

Response of T Cell Clones to TPO Microsomes and to NP-7

5

	<u>Clone</u>	<u>Antigenic Preparation</u>		
		<u>Control Microsome</u>	<u>TPO Microsome</u>	<u>APC</u>
10	c25	79 ± 13	8024 ± 1144	123 ± 32
	c39	175 ± 22	13824 ± 1556	236 ± 19
	c65	54 ± 11	1203 ± 111	70 ± 4
	c69	78 ± 10	3757 ± 517	167 ± 4
	c103	75 ± 12	4575 ± 479	76 ± 12
15	c43	654 ± 396	2121 ± 554	82 ± 12
	c75	84 ± 15	151 ± 30	346 ± 107
	c104	44 ± 6	260 ± 26	68 ± 14
	c105	172 ± 30	1028 ± 141	599 ± 59
20	c3	71 ± 11	78 ± 20	75 ± 11
	c9	72 ± 4	452 ± 32	71 ± 4
	c18	63 ± 3	62 ± 10	49 ± 6
	c20	126 ± 21	704 ± 89	102 ± 9
	c29	121 ± 27	156 ± 17	107 ± 7
25	c60	197 ± 110	345 ± 84	310 ± 53
	c64	50 ± 3	160 ± 13	86 ± 20
	c70	76 ± 17	138 ± 23	93 ± 20
	c77	61 ± 8	645 ± 284	94 ± 11
	c82	1844 ± 143	4246 ± 176	8318 ± 191
30	c83	192 ± 44	139 ± 26	130 ± 13
	c94	95 ± 8	114 ± 17	70 ± 6
	c95	44 ± 6	89 ± 9	62 ± 25
	c98	87 ± 9	96 ± 14	88 ± 8
	c100	99 ± 15	81 ± 10	252 ± 10
40	Responses of thyroid-derived T cell clones to TPO microsomes and peptide NP-7. NP-7 (10 $\mu$ g/ml) or control or TPO microsomes (0.5 to 1 $\mu$ l) were added per well as indicated. Autologous irradiated PBL or EBV-transformed B cells were used as antigen presenting cells (APC) at an APC:T cell ratio of between 2 and 5. Results are the mean cpm ( $\pm$ S.E.M.) of triplicate wells. Positive results were confirmed in 2 to 7 different experiments.			

45

Clones were further screened using a panel of 10 synthetic peptides based on the TPO sequence, selected using two T cell motif algorithms (Rothbard, J.B., Ann. Inst. Pasteur **137E**:518-526 (1986); DeLisis, C. et al., Proc. Natl. Acad. Sci. USA **82**:7048-7052 (1985)) as shown in Table II. Two clones (c43 and c105) which showed only a small response to TPO microsomes (Table IV) showed a specific response to a peptide (NP-7) corresponding to residues 535-551 of TPO (Table III and IV). Two additional clones (c75 and c104), unresponsive to the whole TPO microsome preparation, showed significant responses to NP-7. In contrast, the 5 clones highly reactive to TPO microsomes (c25, c39, c65, c69, c103) did not respond to NP-7 (Table IV). No response to NP-7 was seen with the patients' peripheral blood T cells (PBL alone = 609 ± 190 cpm; PBL + NP-7 (10 µg/ml) = 302 ± 38 cpm).

### C. DISCUSSION

The lack of recognition of NP-7 by TPO responsive clones suggests the presence of additional T cell epitopes on TPO distinct from NP-7. The observation that clones specific for an epitope derived from the TPO sequence (NP-7) are present at high frequency in the thyroid infiltrate, and yet respond poorly or not at all to whole TPO presented by APC of peripheral blood origin, is noteworthy.

These results provide the first clear evidence in human organ-specific autoimmunity that a significant proportion of activated T cells infiltrating the target tissue recognize an antigenic protein specific to that tissue. This is consistent with the finding of collagen type II-specific T cells in the joint in rheumatoid arthritis (Londei, M. et al., Proc. Natl. Acad. Sci. USA **86**:636-640 (1989)). These results also define the site of a T cell epitope within TPO (residues 535-551) and provide evidence for the presence of at least two distinct epitopes on a single target molecule in the same individual.

METHODS

TPO cDNA fragment library construction: A full-length (3.05 kb) cDNA clone as described above for human thyroid peroxidase was released from its Bluescript vector (Stratagene, San Diego, CA.) by digestion with EcoRI (BRL Laboratories, Gaithersburg, MD) and NotI (Boehringer, Mannheim, West Germany). Because both vector and insert are of similar length, the Bluescript was further digested with ScaI (New England Biolabs, Beverly, MA.). The TPO cDNA was purified by agarose gel electrophoresis and electroelution. The cDNA was then digested (6 minutes at room temperature) into small random-sized fragments with DNAase I (0.1 ng DNase/ug cDNA) (BRL) in 20 mM Tris-HCl, pH 7.5, 1.5 mM MnCl<sub>2</sub> and bovine serum albumin, 100 ug/ml. After electrophoresis in 2% SeaPlaque agarose (FMC Bio Products, Rockland, ME), TPO cDNA fragments 200-500 b.p. in length were recovered by electroelution. The ends of the fragments were blunted with the Klenow fragment of DNA polymerase I, and ligated to EcoRI linkers (GAATTCGGCACGAG) containing a nonphosphorylated EcoRI cohesive end and a phosphorylated blunt end (Pharmacia, Piscataway, NJ). After phosphorylation with polynucleotide kinase, excess linkers were removed by electrophoresis in 2% SeaPlaque agarose. The linker-ligated cDNA was again size-selected (200-500 b.p.), electroeluted, ethanol precipitated and ligated into EcoRI-cut lambda-Zap vector (Stratagene). After packaging (Giga-Pak Gold, Stratagene), the library was amplified in XL1-blue cells (Stratagene). cDNA insert sizes were confirmed by the polymerase chain reaction (PCR) (Saiki, R.K., et al., Science 239:487-491 (1988)) using the Bluescript reverse and -20 primers. PCR analysis of the "C2" hTPO cDNA region (Libert, F., et al., EMBO J. 6:4193-4196 (1987); Ludgate, M., et al., J. Clin. Endocrinol. Metab. 68:1091-1096 (1989)) in the TPO cDNA fragment library was performed using two oligonucleotide 22-mer primers (5'-

-70-

GGTTACAATGAGTGGAGGGAGT and 5' - GTGGCTGTTCTCCCACCAAAAC) spanning the region 1852 -2112 b.p. in hTPO (17). PCR (30 cycles) was for 1 minute at 94°C, 2 minutes at 55°C and 1 minute at 72°C. For screening the library, the PCR-generated DNA was labeled with  $^{32}\text{P}$ - $\alpha$ CTP to a specific radioactivity of  $0.8 \times 10^9$  cpm/ $\mu\text{g}$  DNA using the random primer method (Multiprime; Amersham, Arlington Heights, IL). The screening procedure employed standard techniques (Maniatis, T., *et al.*, Molecular Biology: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)), with final washes of 30 minutes (x 2) at 55°C in 0.1 x SSC, 1% SDS buffer (1 x SSC in 150 NaCl, 15 mM Na citrate, pH 7.5). Autoradiography of the nitrocellulose filters was performed with Kodak XAR-5 film.

15            Immunological screening of the TPO sub-library: The lambda-Zap library containing TPO cDNA fragments, plated in E. coli Y1090 at about  $3 \times 10^4$  pfu per 150 mm diameter Petri dish, was screened as previously described (Seto, P., *et al.*, J. Clin. Invest. 80:1205-1208 (1987)). In brief, after 3.5 hours at 42°C, nitrocellulose filters soaked in 10mM isopropyl-thio-beta-D-galactopyranoside (IPTG) were overlayed for 3.5 hours at 37°C. Filters were removed, washed in TBS buffer (10 mM Tris HCl, pH 7.5, 150 mM NaCl) containing 0.05% Tween, incubated for 15 minutes at room temperature in TBS/Tween containing 2% Carnation milk, rinsed with TBS/Tween, and then incubated overnight at 4°C with antibody. For immunological screening, mouse monoclonal antibody (#20.10) against the thyroid microsomal antigen (Portmann, L., *et al.*, J. Clin. Invest. 81:1217-1224 (1988)) was used at a 1:200 dilution. Because of the very low background and strong signal achieved with monoclonal antibodies, pre-adsorption with bacterial proteins is not necessary prior to screening, as previously described (Seto, P., *et al.*, J. Clin. Invest.

Such information is very important for the design of appropriate peptide-based immunotherapy, as discussed above.

EXAMPLE XIII

5           Molecular Determination of a B Cell Epitope of TPO

To determine precisely, at the amino acid level, the epitopes in human TPO that are recognized by antibodies in the sera of patients with autoimmune thyroid disease, a panel of mAbs generated against natural TPO was studied. The binding of some of these mAbs to TPO was inhibited by patients' sera, and determination of the TPO epitopes recognized by these mAb would, indirectly, define the disease-associated epitope(s).

10           This panel of 13 mAbs was used to screen a lambda-Zap library constructed to contain, exclusively, 200-500 bp random fragments of TPO cDNA. When expressed as bacterial fusion proteins, 1/6 of the  $3.8 \times 10^6$  cDNA fragments would express random 66-166 amino acids fragments of TPO.

15           For screening, binding of murine anti-TPO mAb (1:40 dilution) was detected using peroxidase-conjugated goat anti-mouse immunoglobulin antibody. Positive plaques were revealed with only one of the thirteen mAb tested (mAb-47). MAb-47 bound TPO with high affinity but did not interfere with the enzymatic activity of TPO. Human anti-TPO autoantibodies strongly inhibited the binding of mAb-47 at 1:20 dilution.

20           The nucleotide sequences of seven randomly selected clones recognized by mAb-47 were determined. All the clones spanned the same region of the TPO cDNA, overlapping in the region of 2180-2171 bp. This region encodes 30 amino acids (at position 698-728) in the TPO protein.

25           Anti-TPO mAb-47 is unique among 13 mAbs tested in that it recognizes a continuous epitope on TPO. The other mAbs presumably recognize discontinuous epitopes. The competitive binding to TPO of mAb-47 and naturally occurring anti-TPO

autoantibodies suggests that mAb-47 defines a natural, disease-associated TPO epitope.

To further elucidate the molecular and cellular basis for the pathogenesis of autoimmune thyroid disease, it will be very important to identify the sites (epitopes) on TPO recognized by the anti-TPO antibodies in Hashimoto's thyroiditis patients. Prior approaches to the examination of this question have included the use of immunological probes (polyclonal or monoclonal anti-TPO antisera) (Libert, F., et al., EMBO J. **6**:4193-4196 (1987); Ludgate, M., et al., J. Clin. Endocrinol. Metab. **68**:1091-1096 (1989); Doble, N.D., et al., Immunol. **64**:23-29 (1988); Ruf, J., et al., Endocrinol. **125**:1211-1218 (1989); Laing, P., J. Clin. Lab. Immunol. **19**:19-23 (1986); Kohno, Y., et al., J. Clin. Endocrinol. Metab. **68**:766-773 (1989)) and limited proteolytic digestion (Yokoyama, N., et al., J. Clin. Endocrinol. Metab. **68**:766-773 (1989)). By these means, several distinct antibody binding regions appear to be present in TPO.

However, TPO is an extremely large antigen (approximately 107 kD), and these techniques have not allowed definition of the precise epitopes involved. The present inventor therefore undertook to screen, with sera from patients with Hashimoto's thyroiditis, a bacteriophage (lambda-Zap) human thyroid cDNA expression library containing large numbers of hTPO cDNA fragments.

Each of these fragments is 200-500 b.p. in length, coding for TPO polypeptides of 66-166 amino acids. The entire hTPO protein comprises 933 amino acids. These TPO polypeptide fragments are expressed as bacterial fusion proteins, so called because the protein is a hybrid of a 10 kD fragment of  $\beta$ -galactosidase linked to the thyroid protein component.

80:1205-1208 (1987)). Antisera from 13 Hashimoto's thyroiditis patients with high titer antimicrosomal antibodies were used under a variety of different conditions at a dilution of 1:200. In contrast to previous experience in screening lambda gt11 libraries with Hashimoto's sera (Hirayu, H., et al., J. Clin. Endocrinol. Metab. **64**:578-584 (1987)), screening of the lambda-ZAP libraries provided very little background with such sera, and, in general, pre-adsorption was not required to reduce this non-specific background. When pre-adsorption was performed, Y1090 proteins were immobilized on nitrocellulose filters. In addition, affinity-purified anti-TPO antibodies, prepared using recombinant hTPO expressed on the surface of Chinese hamster ovary (CHO) cells also were used as immobilized antigen (Kaufman, K.D., et al., J. Clin. Invest. **84**:394-403 (1989)). For this procedure, 1 ml of serum was diluted 1:10 in phosphate-buffered saline (PBS) containing 0.05% Na azide and 1mM phenylmethyl sulfonylfluoride (PMSF). TPO-CHO cells (approximately  $10^8$ ) were resuspended by light trypsinization, diluted in PBS containing 10% calf serum, pelleted (5 minutes at 1,000 x g), and resuspended in the diluted antibody for 1 hour at 4°C. Unbound antibody was removed by pelleting the cells, followed by a rinse in ice-cold PBS. After recovery by centrifugation (5 minutes at 1,000 x g), the cells were resuspended and incubated for 15 minutes at 4°C in 150 mM acetic acid in PBS containing 0.05% Na azide and 1 mM PMSF. NaOH and 1 M Tris, pH 7.5, were added to neutralize the acetic acid, and the cells and particulate material were removed by centrifugation (5 minutes at 1,000 x g, and then for 30 minutes at 100,000 x g, 4°C), leaving the affinity-purified antibody in the supernatant. The efficiency of the affinity purification was approximately 50%, as measured by ELISA (Schardt, C.W., et al., J. Immunol. Methods **55**:155-168 (1982)).

The detection systems for antibody bound to fusion proteins were as previously described (Seto, P., *et al.*, *J. Clin. Invest.* **80**:1205-1208 (1987)), using the following antisera: For the mouse antimicrosomal monoclonal antibody, 5 peroxidase-conjugated, affinity-purified goat anti-mouse IgG (heavy and light chain specific) (Cappel, Organon, West Chester, PA.) at a dilution of 1:300; For the polyclonal human antisera, anti-human IgG (Fc fragment, gamma chain specific) (Cappel) at a dilution of 1:300. Color was 10 developed with 2.8 mM 4-chloro-1-naphthol (Sigma, St. Louis, MO). The quality of the immunological reagents used in the polyclonal antibody screening procedure was confirmed by their ability to generate a strong signal with eukaryotic recombinant hTPO on western blot analysis (Kaufman, K.D., *et 15 al.*, *J. Clin. Invest.* **84**:394-403 (1989)). Positive clones were plaque-purified to homogeneity. Control screening of potentially positive plaques was performed by omitting the first (anti-TPO) antibody in the screening procedure.

20 Nucleotide sequence analysis of selected clones: Plaque-purified lambda-Zap phage were used to generate Bluescript plasmids containing the fragment of TPO cDNA whose respective fusion proteins had been detected by the antisera. This procedure used the helper phage R408 according to the protocol of the manufacturer (Stratagene). Rescued phagemids were used to infect XL1-blue bacteria (Stratagene). Plasmids were prepared from individual colonies (Maniatis, T., *et al.*, Molecular Biology: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)), and the sizes of 25 the cDNA inserts were assessed by digestion with EcoRI. Nucleotide sequencing of selected plasmid cDNA inserts was performed by the dideoxynucleotide termination method (Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA* **74**:5463-5467 (1977)).

30

Nucleotide sequence analysis was performed using the software provided by Bionet.

#### RESULTS

5       Localization of the epitope for a monoclonal antibody against thyroid peroxidase. In order to define the epitope(s) for anti-TPO antibodies in patients with autoimmune thyroid disease, it was first necessary to determine the validity of the immunological screening of a hTPO cDNA fragment sub-  
10 library (Mehra, V., et al., Proc. Natl. Acad. Sci. USA 83:7013-7017 (1986)). For this purpose, a monoclonal antibody generated against the thyroid microsomal antigen (Portmann, L., et al., J. Clin. Invest. 81:1217-1224 (1988)) that had been used successfully in the past to clone this antigen from  
15 a Graves' thyroid cDNA library (Hirayu, H., et al., J. Clin. Endocrinol. Metab. 64:578-584 (1987)) was used. The new TPO cDNA fragment sublibrary constructed contained  $3.8 \times 10^6$  recombinant clones, with an effective (correct orientation and reading frame) size one-sixth of this number. The insert sizes were confirmed to be in the 200-500 b.p. range.  
20

Screening of this library with the anti-microsomal antigen monoclonal antibody yielded 6-12 positive plaques per 1,000 plaques screened. Fourteen positive clones were randomly chosen for partial nucleotide sequencing to delineate the position of their TPO cDNA inserts relative to the entire TPO gene. Twelve of the 14 clones had cDNA inserts of 160-350 b.p. Two clones (U and Y) that had cDNA inserts slightly larger than the expected 500 b.p. maximum were found, upon nucleotide sequencing, to have double cDNA  
25 inserts. As an indication of the success of the procedure, all 14 clones recognized by the monoclonal antibody spanned the same region (746-1,150 b.p.) of the hTPO gene (Magnusson, R.P., et al., Mol. Endocrinol. 1:856-861 (1987)) (Figure 18). The maximum region common to all clones, and therefore an  
30

indication of a common epitope, was between bases 881 and 927 (AA AAC CCA TGT TTT CCC ATA CAA CTC CCG GAG GAG GCC CGG CCG GCC), corresponding to a derived amino acid sequence of only 15 residues (Asn Pro Cys Phe Pro Ile Gln Leu Pro Glu Glu Ala Arg Pro Ala). Therefore, the epitope recognized by the monoclonal antibody lies within this 15 amino acid span.

5                   Epitope(s) for the antimicrosomal/TPO antibodies in autoimmune thyroid disease. Approximately forty screenings of the same TPO cDNA fragment sub-library described above 10 with sera from patients with Hashimoto's thyroiditis did not yield any positive clones. The modifications that were tried included: 1) the use of different host bacteria (BB4, XL blue and Y1090) in which to express the TPO fusion proteins; 15 2) variation in the antibody binding detection system, including the use of anti-human IgG antibody or protein A from different vendors, as well as different incubation times and temperatures; and 3) the use of thirteen different patients' sera with potent anti-TPO activity. The sera were tested in multiple ways: without bacterial pre-adsorption; 20 following adsorption with bacterial lysate; or after affinity-purification with recombinant hTPO. As internal controls in the screening procedure, the monoclonal antibody always yielded the expected number of positive clones.

25                   Quite surprisingly, it was not possible to detect the epitope expressed within the 86 amino acid C2 hTPO polypeptide fragment, as previously reported (Libert, F., et al., EMBO J. **6**:4193-4196 (1987); Ludgate, M., et al., J. Clin. Endocrinol. Metab. **68**:1091-1096 (1989)). Because of the possibility that the fragment library employed might lack the 30 C2 region, C2 region presence was tested by PCR, using oligonucleotide primers complementary to each end of the C2 region. A fragment of the expected size (261 b.p.) was clearly detected. Further, by using this PCR-generated fragment as a probe to screen the library, it was determined

that approximately 10% of the plaques in the library contain C2 sequence.

Because of these negative results with the Hashimoto's thyroiditis sera in the hTPO cDNA fragment library, these 5 sera also were used to screen lambda-Zap Graves' thyroid libraries (both oligo-dT and random-primed), constructed as described previously (Kaufman, K.D., et al., *J. Clin. Invest.* 84:394-403 (1989)). The oligo-dT-primed library contains numerous full-length copies of TPO cDNA (3.1 kb), as was demonstrated by the ability to express enzymatically active, 10 antigenically intact TPO, when such cDNA was subcloned from the phage vector into a eukaryotic expression plasmid, and stably-transfected into eukaryotic Chinese hamster ovary cells (Kaufman, K.D., et al., *J. Clin. Invest.* 84:394-403 (1989)). 15 Despite this, no specific signal was detected in screening this lambda-Zap library with 13 potent Hashimoto's sera that strongly react immunologically with TPQ expressed in eukaryotic cells (Kaufman, K.D., et al., *J. Clin. Invest.* 84:394-403 (1989)). Many strongly reacting plaques were 20 observed in these screenings, in which plaques reacted with the second antibody (anti-human IgG) even in the absence of patients' serum. Similar findings were obtained in the past with a Graves' thyroid cDNA library in Iambda gt11 (Hirayu, H., et al., *J. Clin. Endocrinol. Metab.* 64:578-584 (1987)). 25 These clones may represent IgG present in B-lymphocytes in the Graves' thyroid gland from which the library was made.

A potential difficulty with protein expression in a full-length cDNA phage library is that stop codons in the 5'-untranslated region of the cDNA insert may interrupt the 30 translation of the foreign protein, which is inserted downstream of the  $\beta$ -galactosidase portion of the fusion protein. To eliminate this possibility, two additional approaches were attempted. The first was screening of a random-primed human thyroid cDNA lambda-ZAP library,

constructed in the same manner as the oligo-dT primed library, with the exception that random primers, rather than oligo-dT, were used for first strand cDNA synthesis. This library contains cDNA clones with a bias against full-length 5 cDNA copies. The second approach was to delete the 5'-untranslated region from the full-length hTPO cDNA clone in the Bluescript plasmid generated from the lambda-Zap clone (Kaufman, K.D., *et al.*, *J. Clin. Invest.* **84**:394-403 (1989)). This deletion was accomplished by digestion of this plasmid 10 with XbaI, thereby releasing 154 b.p. of the 5'-end of hTPO cDNA, leaving the entire TPO protein (minus the signal peptide) remaining in reading frame with the  $\beta$ -galactosidase component of the Bluescript plasmid. This new plasmid construct was transfected into XL1-Blue host bacteria for 15 fusion protein generation (Stratagene, San Diego CA) and western blot analysis. Neither the random-primed library nor the XbaI deletion mutant generated a hTPO protein that could be recognized by Hashimoto's antisera, or with anti-TPO antibody affinity-purified from these sera using recombinant 20 hTPO.

#### DISCUSSION

The present data provide the first definition, at a precise molecular level, of an epitope recognized by an 25 antibody against a thyroid autoantigen. Previous studies using polyclonal or monoclonal antibodies against human thyroglobulin (Male, D.K., *et al.*, *Immunol.* **54**:419-426 (1985); Fukuma, N., *et al.*, *Immunol.* **67**:129-131 (1989)) or TPO (Libert, F., *et al.*, *EMBO J.* **6**:4193-4196 (1987); Ludgate, M., 30 *et al.*, *J. Clin. Endocrinol. Metab.* **68**:1091-1096 (1989); Doble, N.D., *et al.*, *Immunol.* **64**:23-29 (1988); Laing, P., *J. Clin. Lab. Immunol.* **19**:19-23 (1986); Kohno, Y., *et al.*, *J. Clin. Endocrinol. Metab.* **68**:766-773 (1989); Yokoyama, N., *et al.*, *J. Clin. Endocrinol. Metab.* **68**:766-773 (1989)) have

DNA sequences of independent clones were aligned with the TPO cDNA sequence to localize the minimum region of overlap that encompasses the epitope (Mehra, V., et al., Proc. Natl. Acad. Sci. USA 83:7013-7017 (1986)).

5

Western Blots: Recombinant human TPO stably expressed by Chinese hamster ovary cells was used as antigen. Cells were cultured, scraped into buffer containing 10 mM Tris (pH 7.4), 0.25 M sucrose, 2 mg/mL bacitracin, 1 mM phenylmethylsulfonyl-fluoride, 0.1 mM N- $\alpha$ -p-tosyl-L-lysine-chloromethylketone, and 0.1 mM leupeptin (all from Sigma Chemical Co., St. Louis, MO), and a microsomal fraction was prepared, all as previously described. The protein concentration was determined by the method of Bradford (Bradford, M.M., Anal. Biochem. 72:238-254 (1976)). Samples (-100  $\mu$ g protein) were treated with 2% sodium dodecyl sulfate and 5%  $\beta$ -mercaptoethanol (final concentrations) and subjected to 7.5% polyacrylamide gel electrophoresis (Laemmli, U.K., Nature 227:680-685 (1970)). Proteins were transferred to a ProBlot membrane (Applied Biosystems, Foster City, CA) using the MilliBlot transfer system (Millipore Co., Bedford, MA) according to the manufacturer's recommendations. Membranes were processed as previously described, with minor modifications. Incubations with MAb (1:1000 dilution) were performed overnight at 4°C. MAb binding was detected with horseradish peroxidase-linked sheep antimouse immunoglobulin G F(ab')2 (Amersham International, Aylesbury, Buckinghamshire, United Kingdom) diluted 1:1,000 using 0.5 mg/mL 4-chloro-1-naphthol, 0.57 mg/mL imidazole, 17% methanol, and 0.42% hydrogen peroxide as substrate.

## RESULTS

Of the 13 mouse MAbs generated against nondenatured human TPO (Ruf, J., et al., *Endocrinology* 125:1211-8 (1989)), only 1 (no. 47) recognized TPO protein fragments expressed by the cDNA library. The nucleotide sequences were determined for 18 5 randomly selected cDNA clones. All cDNA inserts spanned the same region of the TPO cDNA sequence (Figure 19). The minimal region common to all cDNA fragments was between basepairs 2219 and 2247 of the human TPO cDNA nucleotide sequence, coding for 9 amino acids (residues 713-721) in the protein. These 10 nine amino acids thus represent at least a part of the epitope for anti-TPO monoclonal antibody 47. The inability of the other 12 TPO MAb to recognize TPO peptide fragments expressed by the library could not be attributed to technical difficulties in the screening procedure, because internal 15 controls, TPO MAb 47 and TPO MAb 20.10 (Portmann, L., et al., *J. Clin. Invest.* 81:1217-1224 (1988)) all were strongly positive.

To compare the reactivity of the panel of 13 MAb to TPO fragments generated by the cDNA library (see above) with 20 reactivity to the entire TPO protein, Western blot analyses were performed using these MAb as probes and recombinant human TPO expressed in CHO cells as antigen. For the TPO fragments, only MAb 47 reacted with the entire TPO molecule under denaturating and reducing conditions (Figure 20). As a 25 control, TPO MAb 20.10 (Portmann, L., et al., *J. Clin. Invest.* 81:1217-1224 (1988)), generated against the denatured protein and previously shown to recognize a linear epitope between TPO amino acids 266-281 (Finke, R., et al., *J. Clin. Endocrinol. Metab.* 71:53-59 (1990)), also detected a protein of similar 30 size. Consistent with previous enzyme-linked immunosorbent assay data (Ruf, J., et al., *Endocrinology* 125:1211-8 (1989)), all 13 MAb against native TPO immunoprecipitated nondenatured recombinant human TPO.

suggested that these antibodies recognize different regions of the antigen, but no study has been able to localize an epitope to a region of the molecule as small as 15 amino acid residues in size. The minimum size of a B-cell (antibody-recognized) epitope is under discussion, but is believed to be on the order of 5-10 amino acid residues (Van Regenmortel, M.H.V., *et al.*, Immunol. Lett. 17:95-108 (1988)). Therefore, the 15 residue span of the present invention is very close to the size of the epitope itself.

A remarkable finding in this example is the striking contrast between the positive results with the anti-microsomal/TPO monoclonal antibody, and the inability of naturally-occurring, disease-associated anti-TPO antibodies to recognize the 66-166 amino acid TPO fragments expressed in the library employed. Unlike more linear T-cell epitopes, naturally occurring B-cell epitopes may be more conformational, and subject to influence by the secondary or even tertiary structure of the molecule. Disulfide bonds and contiguity of loops of the folded protein that may be far distant in its linear structure, may contribute to the formation of a B-cell epitope. The present data suggest that the epitope(s) for the disease-associated anti-TPO antibodies are highly conformational.

25

#### EXAMPLE XIV

##### Further Determination of the $\beta$ Cell Epitope on TPO

This example provides an important step in understanding the pathogenesis of Hashimoto's thyroiditis by defining the epitope recognized by antithyroid peroxidase (anti-TPO) antibodies. In Example XIII, a human TPO cDNA sublibrary was constructed expressing random fragments of the protein (each 66-166 amino acids in length) (Mehra, V., *et al.*, Proc. Natl. Acad. Sci. USA 83:7013-7017 (1986)). However, serum from

patients with Hashimoto's disease with high titers of anti-TPO antibodies failed to recognize any of these TPO protein fragments. In contrast, TPO fragments in this library were recognized by a mouse monoclonal antibody (MAb) against 5 denatured human TPO. These data support previous evidence (Hamada, N., *et al.*, *J. Clin. Endocrinol. Metab.* **64**:230-238 (1987); Nakajima, Y., *et al.*, *Mol. Cell. Endocrinol.* **53**:15-23 (1987)) that the disease-associated TPO epitopes are highly conformational and are likely to be formed by noncontiguous 10 (discontinuous) regions of the linear amino acid sequence.

This example presents the determination of the disease-associated B-cell epitopes on TPO, using a panel of 13 MAb generated against nondenatured human TPO (Ruf, J., *et al.*, *Endocrinology* **125**:1211-8 (1989)). The binding of some of 15 these MAb to native TPO is inhibited by anti-TPO antibodies in the serum of patients with autoimmune thyroid disease (Ruf, J., *et al.*, *Endocrinology* **125**:1211-8 (1989)), indicating that these particular MAb epitopes correspond to or are in the vicinity of the disease-associated epitopes. Determination of 20 the epitopes for some of the TPO MAb in the panel could, therefore, delineate molecular domains of the autoimmune thyroid disease-associated B-cell epitopes.

#### MATERIALS AND METHODS

**TPO Fragment Library:** The construction of the TPO random fragment cDNA library ( $3.8 \times 10^6$  plaque-forming units) has been described previously. Immunoscreening of the library was performed by standard techniques, as previously described, 25 using 13 mouse MAb generated against native human TPO (Ruf, J., *et al.*, *Endocrinology* **125**:1211-8 (1989)). Positive clones were plaque-purified and used to generate Bluescript plasmids for nucleotide sequencing of the cDNA inserts (Sanger, F., *et al.*, *Proc. Natl. Acad. Sci. USA* **74**:5463-5467 (1977)). The

DISCUSSION

The present data demonstrate that only one (no. 47) of a panel of 13 MAb generated against native human TPO reacts with random 66-166 amino acid fragments of the 933-amino acid TPO molecule. Consistent with this observation, only MAb 47 recognizes intact TPO after denaturation and reduction, although all 13 MAb in this panel recognize native nondenatured human TPO (Ruf, J., et al., *Endocrinology* 102:1211-8 (1989)). In agreement with our findings, MAb 47 is unique in this panel of TPO Mab, in that it was the only MAb whose binding to TPO could not be abolished by dithiothreitol treatment of the protein. The epitope for TPO MAb 47 (amino acids 713-721) is different from that for TPO MAb 20.10 (amino acids 266-281). Furthermore, TPO MAb 20.10 reacts only with denatured TPO (Portmann, L., et al., *J. Clin. Invest.* 81:1217-1224 (1988)).

Our findings reinforce the emerging concept that many B-cell epitopes are conformational and are likely to be discontinuous. By this it is meant that epitopes on globular proteins are dependent on 3-dimensional structure and consist of a number of different regions of the linear protein brought into apposition by protein folding. Thus, only 1 of 13 MAb generated by immunizing mice with native TPO recognizes a linear epitope expressed in a TPO fragment library or after unfolding of TPO by denaturation and reduction. Because MAb 47 also recognizes the native TPO protein, amino acids 713-721 must be situated on the surface of human TPO (unlike amino acids 266-281 recognized by TPO MAb 20.10). Other contiguous loops in the folded protein derived from different regions of the linear sequence may also contribute to the epitope for MAb 47. Amino acids 713-721 may be the minimum needed for recognition by the antibody.

The binding of TPO MAb 47 to human TPO is inhibited by anti-TPO antibodies in the serum of patients with autoimmune thyroid disease (Ruf, J., et al., *Endocrinology* 125:1211-8 (1989)). Therefore, the linear nine-amino acid (residues 713-721) epitope for MAb 47 either corresponds or is close to an autoantibody-associated TPO B-cell epitope. The present data define specific amino acids in a domain containing an epitope for thyroid autoantibodies. Competition studies with MAb 47 (Ruf, J., et al., *Endocrinology* 125:1211-8 (1989)) suggest that the idiotypic antibody in autoimmune thyroid disease serum that interacts with the MAb 47 epitope is uncommon.

#### EXAMPLE XV

##### Overexpression of Secreted hTPO in Non-Thyroidal Eukaryotic Cells

Previous examples describe expression of recombinant human TPO (hTPO) as both the native, membrane-associated enzyme and as a truncated, secreted protein. In the present example, the overexpression of the secreted form of recombinant hTPO in eukaryotic cells is described. hTPO gene amplification was accomplished with a vector containing the mouse dihydrofolate reductase (dhfr) gene. Stably transfected Chinese hamster ovary (CHO) cells were grown in the presence of progressively increasing concentrations of methotrexate (MTX). TPO expression was measured immunologically in an enzyme-linked immunosorbant assay (ELISA) using anti-TPO antibodies. Attempts to also overexpress the wild-type, membrane-associated form of the enzyme were less successful. While some amplification of the native hTPO gene was observed, it was not possible to achieve a level of protein expression significantly higher than that observed in some high-producing cell lines prior to initiation of selective pressure by MTX. Indeed, above 100 nM MTX, the immunoreactive hTPO content of cells actually

diminished. In contrast, progressive overexpression of the truncated, secreted form of hTPO up to a final MTX concentration of 10,000 nM was observed. Slot-blot analysis of genomic DNA from transfected cells revealed parallel 5 amplification of the dhfr and truncated hTPO genes. High-level expression of secreted hTPO provides a means by which large amounts of biologically and immunologically active hTPO protein may be obtained.

10 MATERIALS AND METHODS

Construction of the expression plasmids pSV2-DHFR-ECE-hTPO and pSV2-DHFR-ECE-hTPO-M1: Full-length hTPO cDNA in the expression vector pECE was digested with PvuI and the ends blunted with the Klenow fragment of DNA polymerase I. The 15 expression vector pSV2-dhfr (kindly provided by Dr. Gordon Ringold, Syntex, Palo Alto, CA) was digested with EcoRI, the ends blunted with Klenow fragment of DNA polymerase I, and the vector treated with bacterial alkaline phosphatase. The blunt-ended, linearized vector and cDNA were ligated together 20 to form the recombinant plasmid pSV2-DHFR-ECE-HTPO. The cDNA coding for the secreted form of hTPO (hTPO-M1), generated in Bluescript by site-directed mutagenesis, was exchanged for wild-type hTPO cDNA in the plasmid pSV2-DHFR-ECE-HTPO to generate pSV2-DHFR-ECE-HTPO-M1.

25 Transfection of pSV2-DHFR-ECE-HTPO and pSV2-DHFR-ECE-HTPO-M1 into CHO dhfr- cells and amplification with methotrexate: CHO dhfr- cells (CHO-DG44; kindly provided by Dr. Robert Schimke, Stanford University, Palo Alto, CA) were maintained 30 in Ham's F-12 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), gentamicin (40 ug/ml) and amphotericin B (2.5 ug/ml). Transfection with plasmid DNA (10 ug) was performed by the calcium phosphate precipitation method (Chen, C., et al., Mol. Cell. Biol. 7:2745-2752 (1987)).

Transfected cells were selected for in thymidine-, guanidine-, and hypoxanthine-free Ham's F-12 medium supplemented with 10% dialyzed fetal calf serum and antibiotics as above. Individual clones were selected with cloning cylinders and 2 clones with high levels of TPO expression (clones CHO-HTPO-2B and CHO-HTPO-C4C) were subsequently used for amplification. Methotrexate (MTX) was added to this selective cell culture medium as an initial concentration of 3.3 nM and surviving cells were harvested and expanded. The methotrexate concentration was sequentially increased by 3.33-fold increments until a final concentration of 10,000 nm (100  $\mu$ M) was reached.

ELISA of CHO-hTPO and CHO-hTPO-M1 cells: ELISA of human sera (kindly provided by Dr. Sandra McLachlan, Cardiff, Wales, UK) of control and MTX-treated CHO-hTPO cells was modified from the method of Schardt *et al.* (*J. Immunol. Methods* 55:155-168 (1982)), as described above, using cellular microsomes. Because the hTPO-M1 protein is secreted into the medium of CHO-hTPO-M1 cells, three-day conditioned media were collected from these cells. Proteins from these media were precipitated and treated, as described above. Antigen for ELISA of human sera was applied as 100  $\mu$ l of the dialyzed protein precipitate per well, approximately 300 ug protein diluted 1:1 in 2 x coating buffer (0.1 M sodium bicarbonate, pH 9.3 + 0.04% sodium azide). Because more than one ELISA was used for all MTX concentrations, values are reported as an ELISA index referenced to 1000 nM MTX values used across assays of each cell type. The same sera were used in ELISAs of each cell type.

Genomic DNA extraction of CHO-hTPO-M1 cells: Cells from confluent 100 mm diameter dishes of CHO-hTPO-M1 cells surviving at each MTX concentration were frozen and kept at

-80°C until replated (100 mm dish), grown to confluence, and used for extraction of genomic DNA. Cells were rinsed three times in 5 ml ice-cold Dulbecco's phosphate-buffered saline, calcium- and magnesium-free (PBS-CMF). The cells were then scraped from the dish, recovered by centrifugation for 10 minutes at 2000 rpm, 4°C. The pellet was resuspended in 2 volumes (100-200  $\mu$ l) 320 mM sucrose, 10 mM Tris-Cl, pH 7.5, 5 mM MgCl<sub>2</sub>, 1% Triton X-100, and kept on ice for 30 minutes. The suspension was centrifuged for 15 minutes at 2500 rpm (4°C), and the pellet resuspended in 4.5 ml 10 mM NaCl, 10 mM Tris-Cl, pH 7.5, 10 mM EDTA. RNase digestion (addition of 4.5  $\mu$ l 10 mg/ml DNase-free RNase for 60 min at room temperature) was followed by proteinase K digestion overnight at 37°C (addition of 0.5 ml 10% SDS + 0.1 ml 10 mg/ml proteinase K). The DNA was then extracted two or three times (until the aqueous phase was clear) with 5 ml 0.1 M Tris-buffered phenol, pH 7.4:CHCl<sub>3</sub>, 4% isoamyl alcohol (1:1), followed by an equal volume extraction with CHCl<sub>3</sub>, 4% isoamyl alcohol. The DNA was precipitated with 0.1 volume 3 M sodium acetate, pH 5.2 and 2 volumes ethanol at -80°C for 2 hours and the pellet resuspended in 0.5 ml TE (10 mM Tris, pH 8.0, 1 mM EDTA). Quality and quantity of genomic DNA samples were assessed by agarose gel electrophoresis and OD at 260 nm. Genomic DNA yield from a 100 mm dish of confluent cells was 20 40-160  $\mu$ g.

Slot blot analysis of CHO-hTPO-M1 cells: Genomic DNA (15  $\mu$ g) from CHO-hTPO-M1 cells was digested with EcoRI, ethanol-precipitated, resuspended in TE buffer, and requantified by 30 OD at 260 nm. Aliquots of this DNA (1.0, 0.5, or 0.25  $\mu$ g) were diluted in 0.5 ml 0.4 N NaOH, 10 mM EDTA, boiled for 10 minutes and placed on ice. Nylon membrane filters (Hybond-N RPN, 3050N, Amersham Corporation, Arlington Heights, IL), rinsed in 0.4 N NaOH, were applied to a slot-blot apparatus

(Minifold II, Schleicher & Schuell, Keene, NH) and the wells were rinsed with 0.5 ml 0.4 N NaOH and vacuum dried. Individual 0.5 ml genomic DNA samples were added per well, vacuum was applied briefly, and the wells were rinsed with 5 0.5 ml 0.4 N NaOH and vacuum dried. The filters were removed, washed briefly in 2x SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) and air dried. Genomic DNA was cross-linked to the filters by UV irradiation (UV Stratalinker 2400, Stratagene, La Jolla, CA), and the filters probed with a 10 labeled, PCR-derived, 0.3 kb fragment of the mouse dhfr cDNA, washed, and autoradiograms performed. Following confirmed removal of first label after boiling in 0.1x SSC (0.015 M NaCl, 0.0015 M sodium citrate), 0.1% SDS for 1 hour, the filters were reprobed with a labeled 0.56 kb fragment of human 15 TPO cDNA, washed, and photographed.

#### RESULTS

Recombinant plasmids pSV2-dhfr-ece-hTPO and pSV2-dhfr-ece-hTPO-M1 were transfected into CHO dhfr- cells to produce 20 CHO-TPO and CHO-TPO-M1 cell lines, respectively. These cell lines were grown in progressively increasing (3.33 fold) MTX concentrations up to 1000 (membrane-associated hTPO) or 10,000 (secreted hTPO), each cycle taking a minimum of three weeks. Cells at each stage of amplification were cryo-preserved and were replated after the final amplification 25 step for comparison of the levels of immunoreactive hTPO expression.

Content of wild type membrane-associated human TPO in microsomal fractions from cell lines CHO-HTPO-25 and CHO-HTPO-C4C was quantitated immunologically by ELISA using anti-TPO antibodies in Hashimoto's thyroiditis serum. In both cell 30 lines, some degree of amplification of TPO immunoreactivity was evident with increasing MTX concentrations, reaching a maximum at 100 nm MTX. This increase was followed by a

gradual fall in immunoreactive TPO protein at higher MTX concentrations up to 1  $\mu$ M. There was a minimal increase of TPO expression in CHO-HTPO-C4C, the cell line with the higher basal (pre-MTX) hTPO content. While there was a greater increment in TPO expression in CHO-HTPO-2B cells, the maximum level achieved was only slightly higher than that in the CHO-HTPO-C4C cells. During MTX-induced gene amplification of both the CHO-HTPO and CHO-HTPO-M1 cells, there appeared to be greater cell death at the 100 to 333 nM MTX step than at lower concentrations, with a delay in growth of surviving cells to confluence.

In contrast to the limited overexpression of TPO with the membrane-associated form of the enzyme, overexpression of the secreted form of hTPO by CHO-HTPO-M1 cells was much greater. In these cells, most of the TPO is secreted into the medium, with little remaining in the cells. TPO expression increased markedly over baseline beginning at 333 nM MTX, with progressive increments up to the highest concentration of used (10  $\mu$ M). Slot-blot analysis of genomic DNA from CHO-HTPO-M1 cells using either a dhfr or hTPO DNA probe revealed similar amplification patterns parallel to that of the pattern of TPO protein expression.

A comparison was made of the amount of TPO available from the membrane-associated (CHO-HTPO-2B cells) and secreted protein (CHO-HTPO-M1 cells) for immunological detection in an ELISA. Three-day conditioned media from a single 100 mm dish of confluent CHO-HTPO-M1 cells (10  $\mu$ M MTX) yielded significantly more TPO protein than did microsomes prepared from a 100 mm confluent dish of CHO-HTPO-2B cells (100 nM MTX). Both of these cell lines represented their highest levels of TPO expression.

EXAMPLE XVIThe Role of Carbohydrate Moieties in Recognition of TPO by Anti-TPO Antibodies in Hashimoto's Thyroiditis

5       Carbohydrate moieties on hTPO may contribute to the epitopes recognized by anti-hTPO antibodies in Hashimoto's thyroiditis. This is because bacterial fusion proteins, unlike proteins expressed in eukaryotic cells, are not glycosylated. Very little is known about the carbohydrate  
10      moieties in hTPO. Human TPO (Ruf, J., et al., Acta Endocrinol.  
         Suppl. 281:49-56 (1987)) and the microsomal antigen (Kajita,  
         Y., et al., FEBS Lett. 187:334-338 (1985)) are bound to the  
lectin concanavalin A. The latter is also partially bound to  
lentil lectin (Kajita, Y., et al., FEBS Lett. 187:334-338  
15      (1985)). It is unknown whether the hTPO carbohydrate structures are N-linked, O-linked, or both. In the present example, the nature of the carbohydrate components of hTPO was examined, and whether or not hTPO carbohydrate plays a role in the structure of naturally occurring epitopes in Hashimoto's  
20      thyroiditis.

METHODS AND MATERIALS

Cell culture, protein radiolabeling and hTPO immunoprecipitation: Chinese hamster ovary (CHO) cells stably expressing human hTPO (CHO-TPO 12g) (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)) were cultured in 100 mm diameter dishes in F12 medium containing 10% fetal calf serum, 100 U/ml penicillin, 40 µg/ml gentamicin and 2.5 µg/ml amphotericin B. For radiolabeling, subconfluent cells were rinsed twice in phosphate-buffered saline without calcium and magnesium (PBS-CMF), and were then incubated for 15-20 minutes in methionine-free F12 medium (3 ml/dish) containing 10% dialyzed fetal calf serum. <sup>35</sup>S-methionine (>1100 Ci/mmol; Amersham, Arlington Heights, IL) was then added to the medium  
25  
30

(0.2 mCi/ml), and the incubation was continued for 2-4 hours at 37°C. The medium was removed and the cells were rinsed twice in PBS-CMF, scraped into ice-cold PBS-CMF, pelleted for 10 minutes at 1000 x g (4°C), washed once in 10 ml of the same buffer, and the cell pellet resuspended (0.3 ml/dish of cells) in homogenization buffer (50 mM Hepes, pH 7.5, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 2 mg/ml bacitracin, 0.25 mM TLCK (N-p-tosyl-l-lysine chloro-methyl ketone) and 0.1 mM leupeptin (Sigma Chemical Co., St. Louis, MO). After shaking for 1 hour at room temperature, the mixture was centrifuged for 1 hour at 100,000 x g (4°C), and the supernatant was diluted to 1 ml in immunoprecipitation buffer (10 mM Na phosphate, pH 7.2, 1 M NaCl, 0.1% Na dodecylsulfate, 0.5% NP-40 and 2 mM EDTA).

15       The 1 ml of solubilized cellular proteins was pre-adsorbed twice for 10 minutes at room temperature with 80 µl of 10% IgG-Sorb (Staphylococcus A) (The Enzyme Center, Malden, MA), followed by removal of the IgG-Sorb by centrifugation for 3 minutes at 10,000 x g in a microfuge. Hashimoto's 20 thyroiditis sera with high titers (ELISA readings >1.5 O.D. units) of anti-hTPO antibodies were added to a final dilution of 1:200. Similar results were obtained with three separate sera. After incubation overnight at 4°C, 150 µl of IgG-Sorb 25 were added, and the tubes rotated end over end for 2-4 hours at room temperature. The IgG-Sorb was recovered by centrifugation for 5 minutes at 10,000 x g, washed 5 times with 1 ml of immunoprecipitation buffer, and then once with 10 mM Tris, pH 7.5, 2 mM EDTA and 0.5% Na dodecylsulfate. Finally, the pellet was resuspended in Laemmli sample buffer 30 (31), with 50 mM dithiothreitol (DTT), boiled for 3 minutes, and applied to 6% polyacrylamide gels. Molecular weight markers (Sigma; St. Louis, MO) were as follows: 205 kD myosin; 116 kD  $\beta$ -galactosidase; 97 kD phosphorylase b; 66 kD

bovine serum albumin; 45 kD ovalbumin. Autoradiography was performed with Kodak XAR-5 film.

Enzymatic deglycosylation of immunoprecipitated human TPO: Recombinant, radiolabeled hTPO, immunoprecipitated and bound to IgG-Sorb, as described above, was recovered in enzymatic digestion buffers rather than in Laemmli sample buffer. Enzymatic digestions (18 hours at 37°C) were as follows: endoglycanase F (Boehringer-Mannheim, West Germany, 30 U/ml; in 100 mM Na phosphate buffer, pH 6.0, 50 mM EDTA, 0.1% SDS, 1% beta-mercaptoethanol and 1% NP40); endoglycanase H (Boehringer, 0.2 U/ml; in the same buffer as for endo F, except that EDTA was omitted); O-glycanase (Boehringer, 2.5 U/ml; same buffer as for endo H); and neuraminidase (Sigma, 1 U/ml; in 100 mM Na acetate, pH 5.2, 5 mM EDTA and 1%  $\beta$ -mercaptoethanol). As a control, to monitor degradation of the hTPO, each experiment included a sample incubated in parallel without added enzyme.

Lectin affinity chromatography: Detergent extracts of CHO-TPO cells (5-7 100 mm diameter dishes) were radiolabeled with  $^{35}$ S-methionine (see above) and applied to 2 ml bed volume columns of Concanavalin A (Con A), peanut agglutinin (PNA), wheat germ agglutinin (WGA), Ricinus communis agglutin 1 (RCA1) and Ulex Europaeus (UEA-F) agarose-bound lectins (all purchased from Vector Laboratories, Burlingame, CA). For application to the columns, samples (0.3 ml) were diluted to 10 ml in Buffer A (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Triton X-100), supplemented with the following for each individual lectin: WGA and RCA1- 1 mM EDTA; Con A - 1mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>; PNA - 1mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>; UEA-F - 1mM CaCl<sub>2</sub>. After application to the columns, the unbound proteins were removed by washing with 50 ml of the foregoing Buffer A's. Specifically adsorbed proteins were eluted with 25 ml of the following (all at 300 mM):- WGA, N-acetylglucosamine; PNA and RCA1, D-galactose; Con A,  $\alpha$ -

methyl-D-mannoside; and UEA-F,  $\alpha$ -fucose. Fractions of 0.5 ml were collected and counted for radioactivity in a liquid scintillation counter. The two fractions containing the peak of the eluted radioactivity were pooled (1 ml) and subjected to immunoprecipitation with anti-hTPO antibodies in the sera of patients with Hashimoto's thyroiditis, followed by polyacrylamide electrophoresis and autoradiography (see above).

10 RESULTS

As described above, the derived amino acid sequence of human TPO (Magnusson, R.P., *et al.*, *Mol. Endocrinol.* 1:856-861 (1987); Kimura, S., *et al.*, *Proc. Natl. Acad. Sci. USA* 84:5555-5559 (1987); Libert, F., *et al.*, *Nucl. Acids Res.* 15:6735 (1987)) suggests that there are 5 potential glycosylation sites in the extracellular domain of the enzyme. This is based on the tri-peptide algorithm for glycosylation sites of Asn-X-Ser/Thr (X refers to any amino acid; the third position can be either Ser or Thr). Carbohydrate chains can be linked to the Asn residue (N-linked), or to the Ser or Thr residues (O-linked).

To determine whether hTPO carbohydrate moieties were N-linked, O-linked, or both, and also to obtain information about the characteristics of the carbohydrate component(s), hTPO was digested with a number of deglycosylating enzymes of varying specificity. To prepare radiolabeled hTPO, proteins in Chinese hamster ovary (CHO) cells expressing recombinant hTPO were radiolabeled with  $^{35}\text{S}$ -methionine, followed by immunoprecipitation with anti-hTPO antibodies present in the serum of patients with Hashimoto's thyroiditis. As observed previously on western blot analysis (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)), recombinant hTPO was present as a doublet of approximately 115 kD and 110 kD, with the relative dominance of the 115 kD and the 110 kD bands

varying from experiment to experiment. Digestion with endoglycosidase (endo) F, which removes both complex and polymannose (Thotakura, N.R., *et al.*, *Meth. Enzymol.* 138:350-359 (1987)) N-linked glycans by cleaving the glycosidic linkage between the two N-acetyl glucosamine (GlcNac) residues in the chitobiose core, increased the electrophoretic mobility of the hTPO doublet to approximately 110 kD and 105 kD. Endo H, which acts similarly to endo F on polymannose but differently from endo F on complex glycans, also converted the mobility of hTPO to a 110 kD and 105 kD doublet. In contrast, O-glycanase and neuraminidase, which remove O-linked glycans and terminal neuraminic acid, respectively, did not alter the mobility of radiolabeled hTPO. These data suggest that human TPO contains only polymannose N-linked glycans.

Lectin affinity chromatography (Merkle, R.K., *et al.*, *Meth. Enzymol.* 138:232-259 (1987)) provided further support for the polymannose nature of the hTPO carbohydrate moieties. Thus, radiolabeled, recombinant hTPO was retained only on concanavalin A-Sepharose, which binds with high affinity to N-linked oligosaccharides in which at least two outer mannose residues are either unsubstituted, or are substituted only at position C-2 by another sugar. Bound hTPO could be eluted with 300 mM  $\alpha$ -methyl-D-mannoside. TPO did not bind to wheat germ agglutinin (specificity for terminal and internal GlcNac and terminal neuraminic acid), Ricinus communis agglutinin I (RCAI) (highest affinity for bi- and tri-antennary N-linked oligosaccharides with terminal galactose residues), peanut agglutinin (terminal Gal- $\beta$ -1,3-GalNac) or Ulex europaeus ( $\alpha$ -L-fucose).

Having determined the type of carbohydrate present in recombinant human TPO, the inventor investigated whether these residues play a role in the disease-associated epitopes on hTPO that are recognized by anti-hTPO antibodies in Hashimoto's thyroiditis.

Radiolabeled recombinant hTPO was first partially purified by concanavalin A-Sepharose affinity chromatography, next digested with three different glycanases, and finally subjected to immunoprecipitation with anti-hTPO antibody in 5 Hashimoto's thyroiditis serum. Complete removal of the N-linked carbohydrate chains distal to the chitobiose core with endo F and endo H did not prevent antibody binding. In view of the complexity of these experiments, it is important to note the completeness of N-glycanase treatment. Thus, after 10 deglycosylation, all of the hTPO immunoprecipitated was as the smaller (110 kD and 105 kD) doublet. As a further control, digestion with O-glycanase led to the immunoprecipitation of an unaltered hTPO form (115 kD and 110 kD).

## 15

DISCUSSION

20

25

30

Previous studies have shown that the thyroid microsomal antigen (Kajita, Y., *et al.*, FEBS Lett. **187**:334-338 (1985)) and immunopurified, non-recombinant human TPO (Ruf, J., *et al.*, Acta Endocrinol. Suppl. **281**:49-56 (1987)) are bound by concanavalin A. However, the present inventor is not aware of other data on the nature of the oligosaccharide (glycan) moieties in human TPO. By taking advantage of the expression of recombinant human TPO in non-thyroidal eukaryotic cells as described hereinabove, the present data provide new information on this subject.

Thus, by both glycan enzymatic digestion and by differential lectin affinity chromatography, the data presented in this example provide strong evidence that all the carbohydrate moieties on hTPO are linked to Asn residues (N-linked) and not to Ser or Thr (O-linked). Furthermore, the selective deglycosylation with endo H (Thotakura, N.R., *et al.*, Meth. Enzymol. **138**:350-359 (1987)), as well as the selective adsorption to concanavalin A (Merkle, R.K., *et al.*,

Meth. Enzymol. 138:232-259 (1987)), suggests that these N-linked oligosaccharides are of the polymannose variety.

5        Most important from the perspective of the pathogenesis of Hashimoto's thyroiditis, the present data indicate that the oligosaccharides present in hTPO do not significantly influence the epitopes recognized by anti-hTPO antibodies in the sera of patients with autoimmune thyroid disease, primarily Hashimoto's thyroiditis.

10      An assumption inherent in the present example is that the glycan components of recombinant hTPO are structurally the same as those in TPO present in human thyroid cells in vivo. While it cannot be excluded that Chinese hamster ovary cells may glycosylate the hTPO polypeptide chain in a manner different from human thyroid cells, it is likely that any 15     such differences would be minor. Thus, unlike the polypeptide glycosylation pattern in yeast and bacteria, glycosylation in eukaryotic Chinese hamster ovary cells would be very similar, if not identical, to that in human thyroid cells. Further support for this assumption is that 20     recombinant hTPO in Chinese hamster ovary cells is functionally active, at the same level present in human thyroid cells in monolayer culture (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)). In addition, virtually all sera from patients with Hashimoto's thyroiditis that contain 25     anti-microsomal antibodies can recognize this form of recombinant human TPO on western blot analysis (Kaufman, K.D., et al., J. Clin. Invest. 84:394-403 (1989)) or by ELISA. Thus, by definition, the recombinant human TPO of the present invention contains the relevant, disease-associated epitopes 30     on hTPO.

The present findings that removal of the carbohydrate moieties on human TPO does not affect the antigenicity of the molecule with respect to recognition by anti-hTPO antibodies in Hashimoto's thyroiditis serum are consistent with data

obtained with tunicamycin (see Example XI). However, the present data are much stronger.

The present data suggest that oligosaccharide components in hTPO are not part of the "natural" epitopes recognized by anti-hTPO antibodies in the sera of patients with autoimmune thyroiditis. However, it remains possible that the glycosylated portion of the molecule could influence the interaction of the antibody with its epitope(s), such as by altering the affinity of this interaction. Although not intending to be bound by any particular theory, there is increasing recognition that the majority of epitopes recognized by both polyclonal and monoclonal antibodies are discontinuous. That is, by folding of the polypeptide chain, the three-dimensional structure of a protein may bring into apposition, as an epitope, widely separated, "discontinuous" regions of the polypeptide chain. This three-dimensional configuration may be lacking in peptide fragments, or may be altered by the  $\beta$ -galactosidase component of the bacterial fusion protein.

The present data relate to the recognition of epitopes on TPO by antibodies (B-cell epitopes). These B-cell epitopes are now recognized to be distinct from epitopes presented to T-cells in a major histocompatibility antigen (MHC) restricted manner (Livingstone, A.M., et al., *Ann. Rev. Immunol.* 5:477-501 (1987)). B-cell epitopes are likely to be important in mediating damage by the immune system to the thyroid gland, while T-cell epitopes are likely to be relatively more important in the initiation of the autoimmune process.

EXAMPLE XVIIIdentification and Sequencing of  $\beta$ -Cell Region Capable of Binding  $\beta$ -Cell Epitope on Thyroid Peroxidase

5

The sequences disclosed by the invention in, inter alia, Examples XIII and XIV provide for a method of identifying the interaction responsible for the  $\beta$ -cell recognition of thyroid peroxidase.

10

In detail, using the sequences disclosed in Examples XIII and XIV of the preferred embodiments, it is possible to isolate the proteins which bind to these sequences. This is accomplished using methods, well known in the art, of purifying a protein which binds to a specific DNA sequence.

15

Preferably, a protein which binds to a specific DNA sequence is purified using affinity chromatography. Specifically, the nine amino acid sequence corresponding to residues 713-721 of thyroid peroxidase is immobilized on an appropriate matrix, such as Sepharose, and used as an affinity matrix to purify

20

the proteins which bind to the particular sequence (Arcangioli B., et al., Eur. J. Biochem. 179:359-364 (1989)).

25

Preferably, the DNA binding protein is extracted from human  $\beta$  cells. The protein extract, obtained from the  $\beta$  cell, is applied to a column which contains the immobilized DNA sequence of interest. Proteins which are not capable of binding to the DNA sequence are washed off the column. Proteins which bind to the DNA sequence are removed from the column using a salt gradient. The proteins eluted from such a column are enriched for the proteins which bind to the specific DNA sequences immobilized on the matrix. The DNA binding protein is further purified using procedures well known in the art, such as ion exchange chromatography, high performance liquid chromatography, size exclusion chromatography, and the like.

30

During the purification of the DNA binding protein, the protein is assayed, for example, using the well known gel

35

retardation assay (Garner, M.M. *et al.*, Nucl. Acid Res. **9**:3047 (1981); Fried, M. *et al.*, Nucl. Acid Res. **9**:6506 (1981)), or other well known methods.

Once the DNA binding protein is purified, a partial  
5 amino acid sequence is obtained from the N-terminal of the protein. Alternatively, the protein is tryptically mapped and the amino acid sequence at one of the fragments is determined by methods known in the art.

10 The deduced amino acid sequence is used to generate an oligonucleotide probe. The encoding sequence can be based on codons which are known to be more frequently used by the organism. Alternatively, the probe can consist of a mixture of all the possible codon combination which could encode the polypeptide. Such methods are well known in the art.

15 A probe complementary to the amino acid sequence is used to screen either a cDNA or genomic library for the genomic sequences which encode the DNA binding protein. Once the gene encoding the DNA binding protein has been obtained, the sequence of the DNA is determined according to well known  
20 methods. The gene can be used to obtain large amounts of the protein from a recombinant host, or the sequence can be used in mutational analysis to further define the functional regions within the protein which interacts with the DNA.

25 Alternatively, proteins which bind to  $\beta$ -cell epitope (residues 713-712) are isolated by identifying a clone expressing the protein using well known techniques such as Southwestern blotting (Sharp, Z.D. *et al.*, Biochim Biophys Acta, **1048**:306-309 (1990); Gunther, C. V. *et al.*, Genes Dev. **4**:667-679 (1990); and Walker, M.D. *et al.*, Nucleic Acids Res. **18**:1159-1166 (1990)).

30 In a Southwestern blot, a labeled DNA sequence is used to screen a cDNA expression library whose expressed proteins have been immobilized on a filter via colony or plaque transfer. The labeled DNA sequences bind to colonies or plaques which

-98-

express a protein capable of binding to the particular DNA sequence. Clones expressing a protein which bind to the labeled DNA sequence are purified and the cDNA insert which encodes the DNA binding protein is isolated and sequenced.

5       The isolated DNA can be used to express large amounts of the protein for further purification and study, used in isolating the genomic sequences corresponding to the cDNA, or used to generate functional derivative of the binding protein.

The present invention is thus directed to DNA binding  
10      proteins which can bind to the  $\beta$ -cell epitope and to functional derivatives thereof.

-99-

CLAIMS

WHAT IS CLAIMED IS:

1. A peptide comprised of the nine amino acid region comprising residues 713-721 of recombinant human thyroid peroxidase, or a functional or chemical derivative thereof.
2. A peptide comprised of the amino acid sequence  
LYS-PHE-PRO-GLU-ASP-PHE-GLU-SER-CYS  
or a functional or chemical derivative thereof.
3. A peptide which binds to the B-cell epitope of thyroid peroxidase.
4. The peptide of claim 3, wherein said peptide is isolated from a B-cell protein.
5. The B-cell protein of claim 4, wherein said protein is recombinantly produced.
6. A peptide comprised of a disease associated B-cell epitope of human thyroid peroxidase.

-100-

7. The DNA sequence encoding the peptide of claims 1-6.

8. Recombinant thyroid peroxidase, wherein a nine amino acid region comprising amino acid residues 713-721 has been deleted or replaced.

9. An antibody against the peptide of claims 1-6.

10. The antibody of claim 9 wherein said antibody is selected from the group consisting of a monoclonal antibody, a polyclonal antibody, an anti-idiotypic antibody and an anti anti-idiotypic antibody.

11. A method of detecting human thyroid peroxidase in a sample, comprising contacting said sample with the antibody of claims 9 or 10, wherein said antibody is detectably labeled, so as to form a complex between the human thyroid peroxidase in said sample and said detectably labeled antibody, and detecting complexed or uncomplexed detectably labeled antibody.

12. A pharmaceutical composition comprising the anti-idiotypic antibody of claim 10.

-101-

13. Use of the pharmaceutical composition of claim  
12 for the treatment of a patient suffering from immune  
disease.

14. Use according to claim 13, wherein said immune  
disease is Hashimoto's thyroiditis.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/06283

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C12N 9/00, 15/52; A61K 37/395; C07K 7/06, 15/28  
 US CL :424/85.8; 536/27; 530/328, 387; 435/183

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.8; 536/27; 530/328, 387; 435/183

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Dialog, APS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Clinical Endocrinology and Metabolism, Volume 71, No. 1, issued 1990, R. Finke et al. "Evidence for the Highly Conformational Nature of the Epitope(s) on Human Thyroid Peroxidase that are Recognized by Sera from Patients with Hashimoto's Thyroiditis", pages 53-59, see the entire document.	1-14
Y	Journal of Clinical Endocrinology and Metabolism, Volume 71, No. 2, issued 1990, Nagayama et al. "Characterization, by Molecular Cloning, of Smaller Forms of Thyroid Peroxidase Messenger Ribonucleic Acid in Human Thyroid Cells as Alternatively Spliced Transcripts", pages 384-390, see the entire document.	1-14
Y	Journal of Endocrinology, Volume 124 (Suppl.), Abst. No. 196, issued 1990, J.P. Banga et al. "Localization of Auto-Antigenic Epitopes on Recombinant Thyroid Peroxidase Preparations", see the entire document.	1-14

 Further documents are listed in the continuation of Box C.

See parent family annex.

*	Special categories of cited documents:	"T"	later documents published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier documents published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

13 October 1992

Date of mailing of the international search report

23 OCT 1992

Name and mailing address of the ISA/  
 Commissioner of Patents and Trademarks  
 Box PCT  
 Washington, D.C. 20231  
 Facsimile No. NOT APPLICABLE

Authorized officer

NANCY T. VOGEL

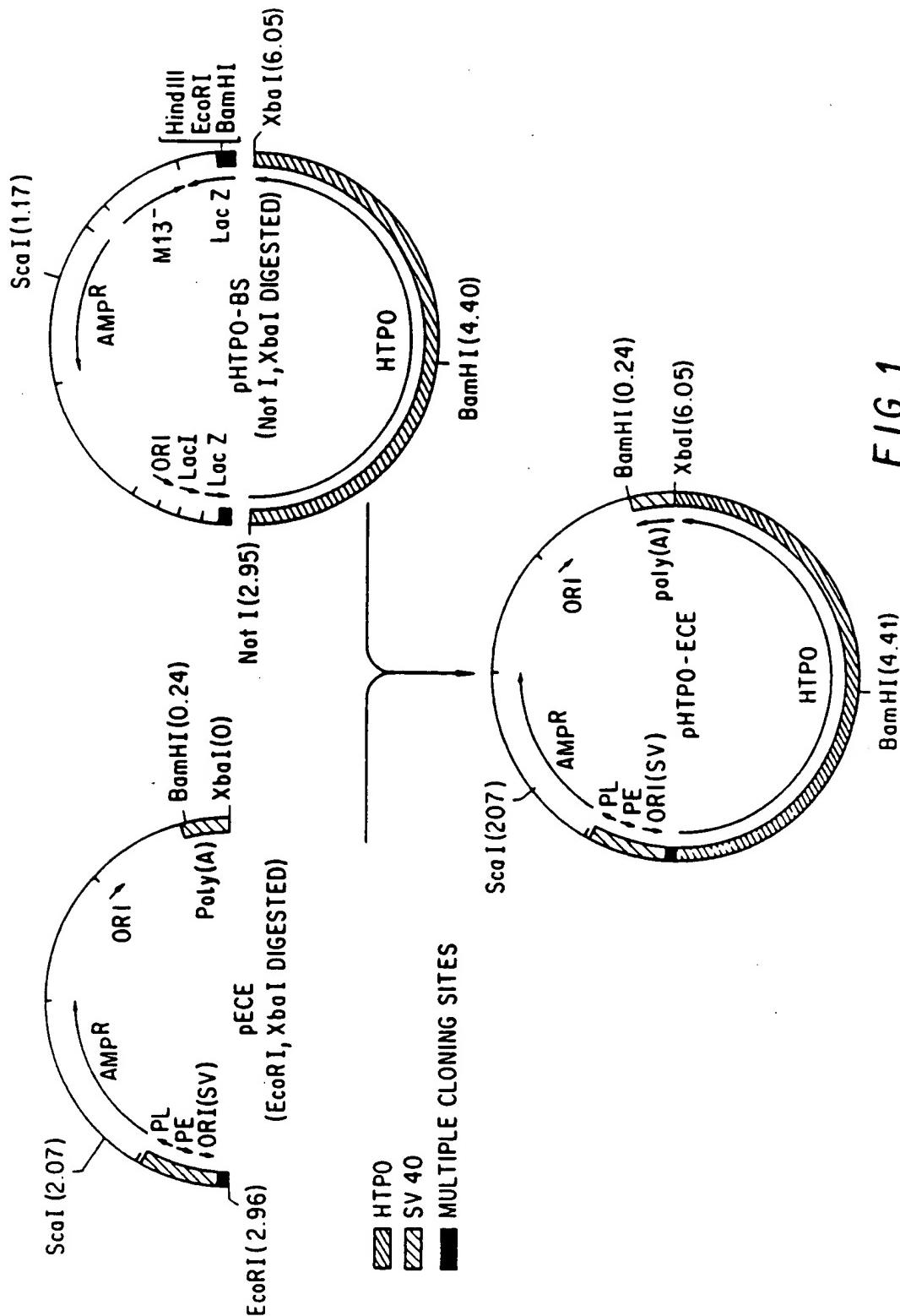
Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/06283

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Endocrinology, Volume 124 (Suppl.), Abst. No. 86, issued 1990, D. L. Ewins et al, "Mapping Epitope Specificities of Monoclonal Antibodies to Thyroid Peroxidase Using Recombinant Antigen Preparations", see the entire document.	1-14
Y	Biochemical and Biophysical Research Communications, Volume 164, No. 3, issued 15 November 1989, J. Hata et al, "Stable High Level Expression of Human Thyroid Peroxidase in Cultured Chinese Hamster Ovary Cells", pages 1268-1273, see the entire document.	1-14
Y,P	Biochemical and Biophysical Research Communications, Volume 179, No. 1, issued 30 August 1991, S. Portolano et al, "A Human FAB Fragment Specific for Thyroid Peroxidase Generated by Cloning Thyroid Lymphocyte-Derived Immunoglobulin Genes in a Bacteriophage Lambda Library", pages 372-377, see the entire document.	1-14
Y	Science, Volume 215, issued 26 March 1982, P. Potocnjak et al, "Inhibition of Idiotype-Anti-Idiotype Interaction for Detection of a Parasite Antigen: A New Immunoassay", pages 1637-1639, see the entire document.	1-14



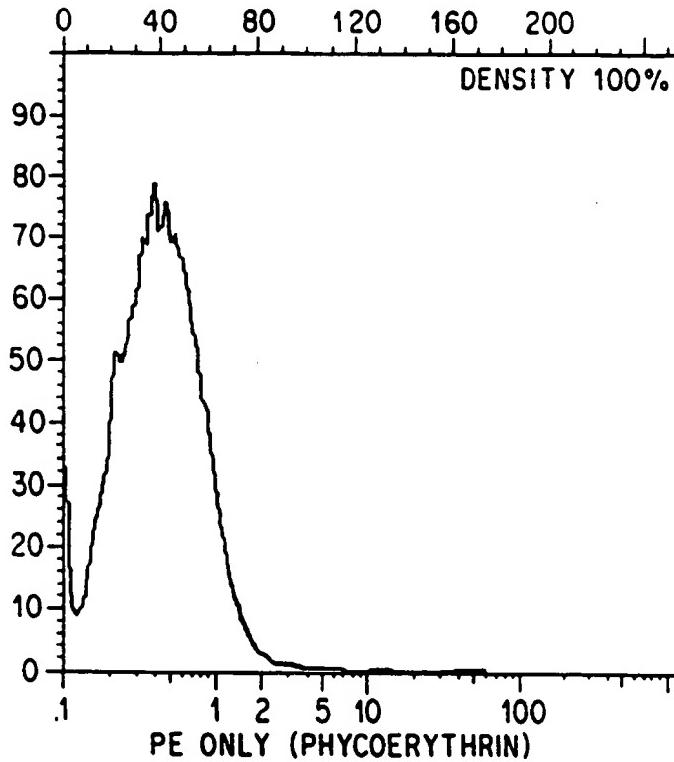
SUBSTITUTE SHEET

FIG. 1

BamHI(4.41)

2/27

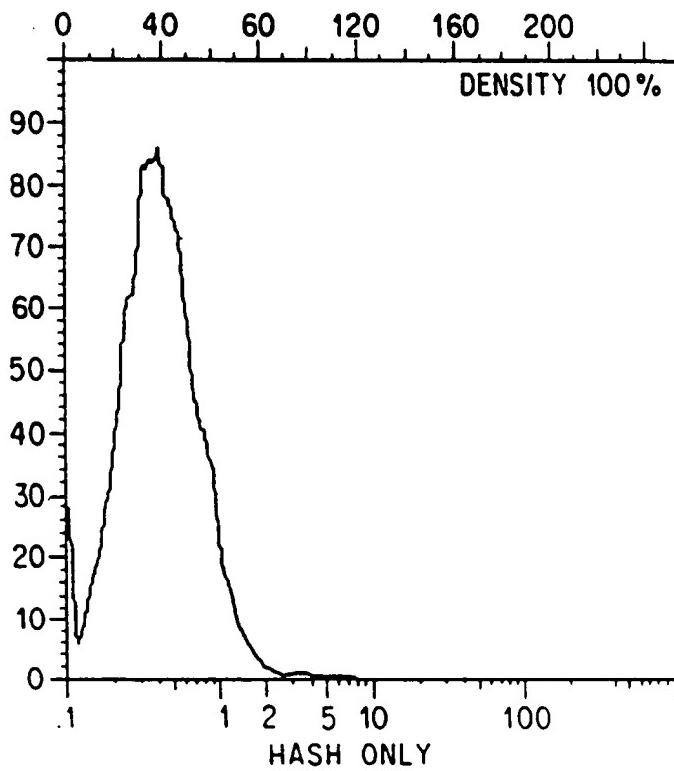
RELATIVE  
NUMBER  
OF CELLS



PE ONLY (PHYCOERYTHRIN)

**FIG. 2A**

RELATIVE  
NUMBER  
OF CELLS



HASH ONLY

**FIG. 2B****SUBSTITUTE SHEET**

3/27

RELATIVE  
NUMBER  
OF CELLS

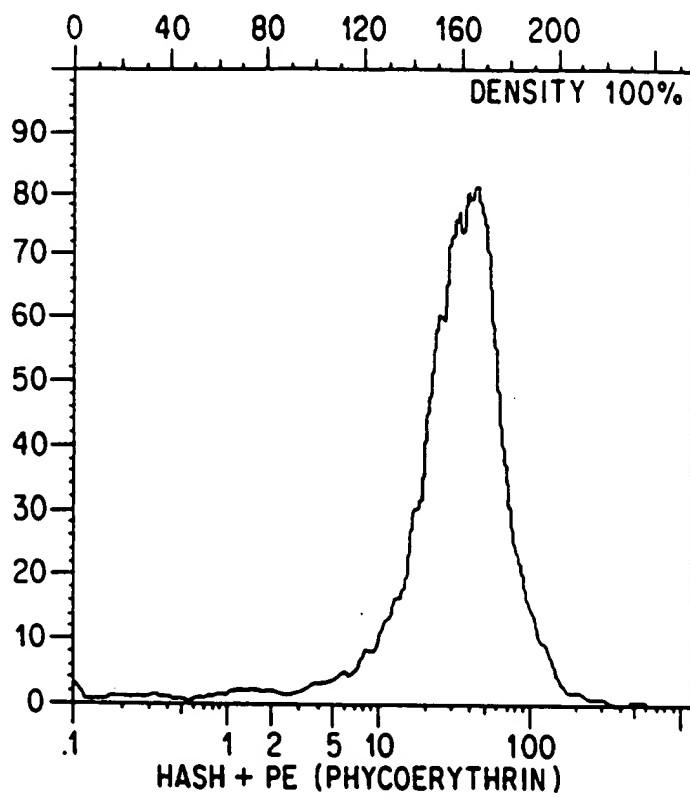
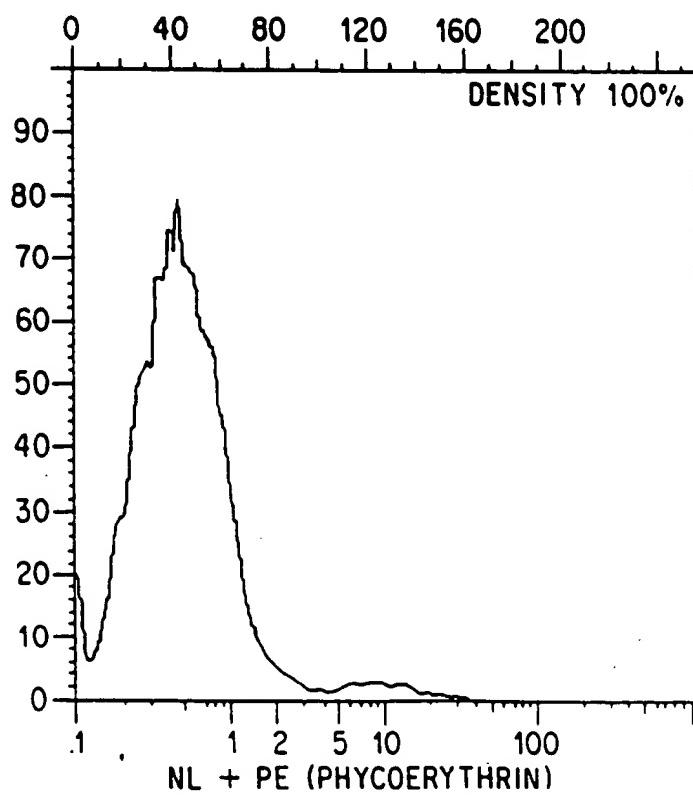


FIG. 2C

RELATIVE  
NUMBER  
OF CELLS



SUBSTITUTE SHEET

4/27

HASH + PE  
(PHYCOERYTHRIN)

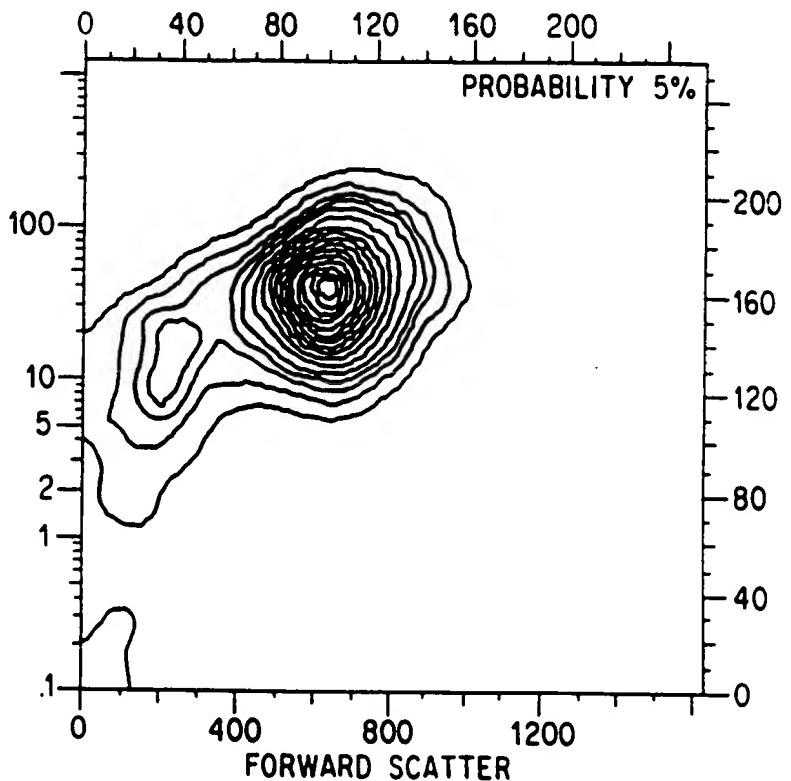
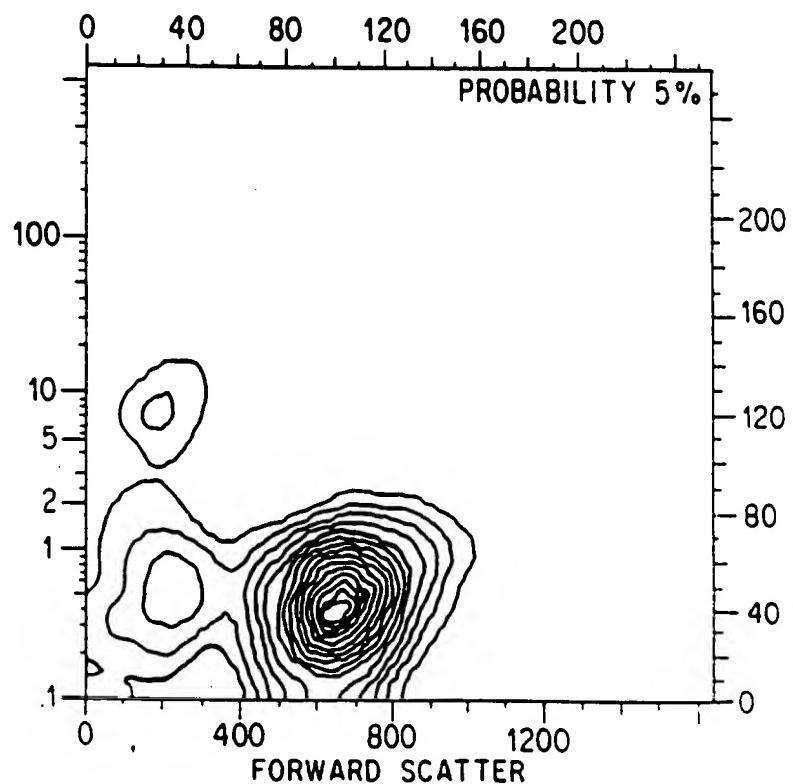


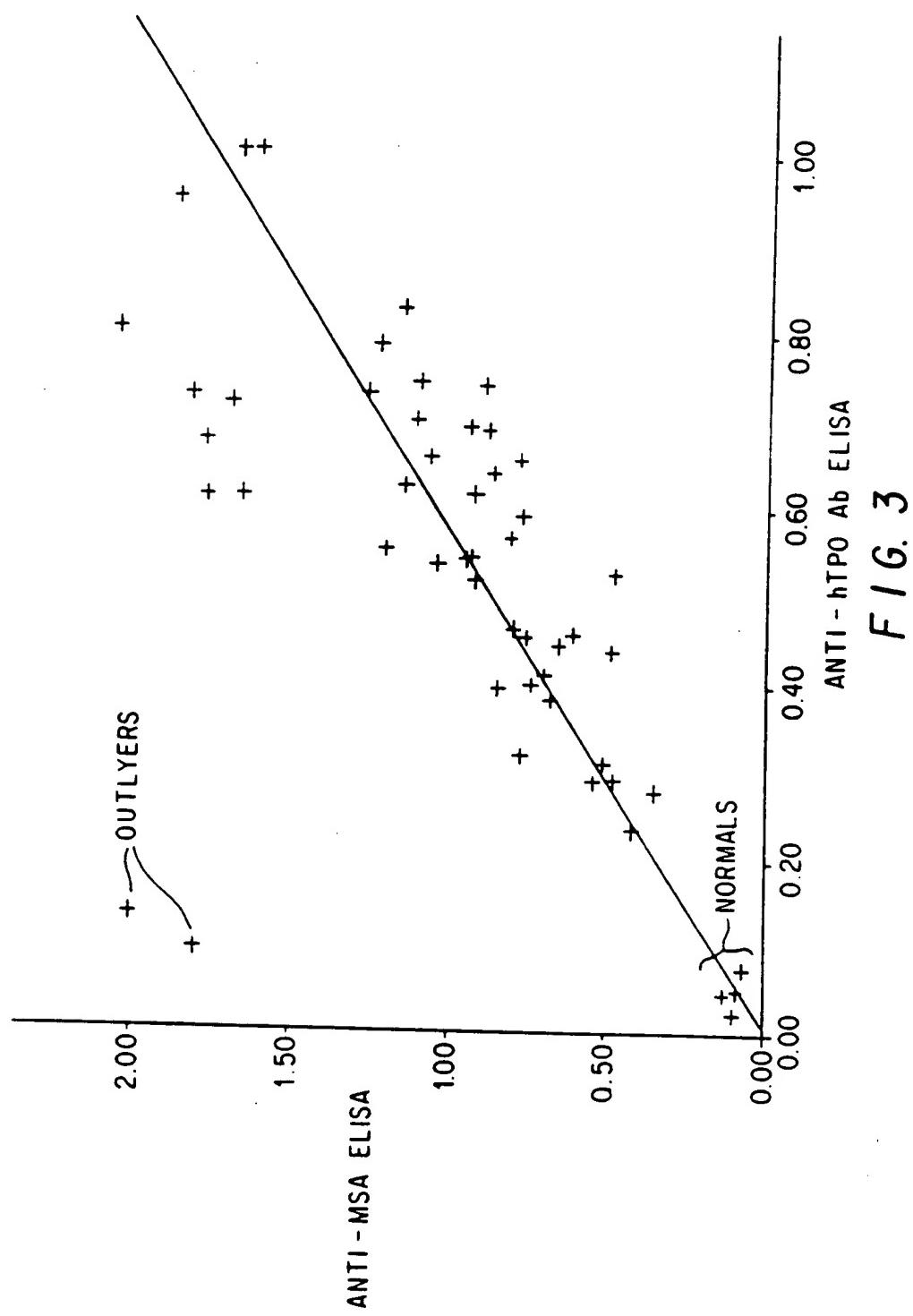
FIG. 2E

NL + PE  
(PHYCOERYTHRIN)



SUBSTITUTE SHEET

5/27

**SUBSTITUTE SHEET**

6/27

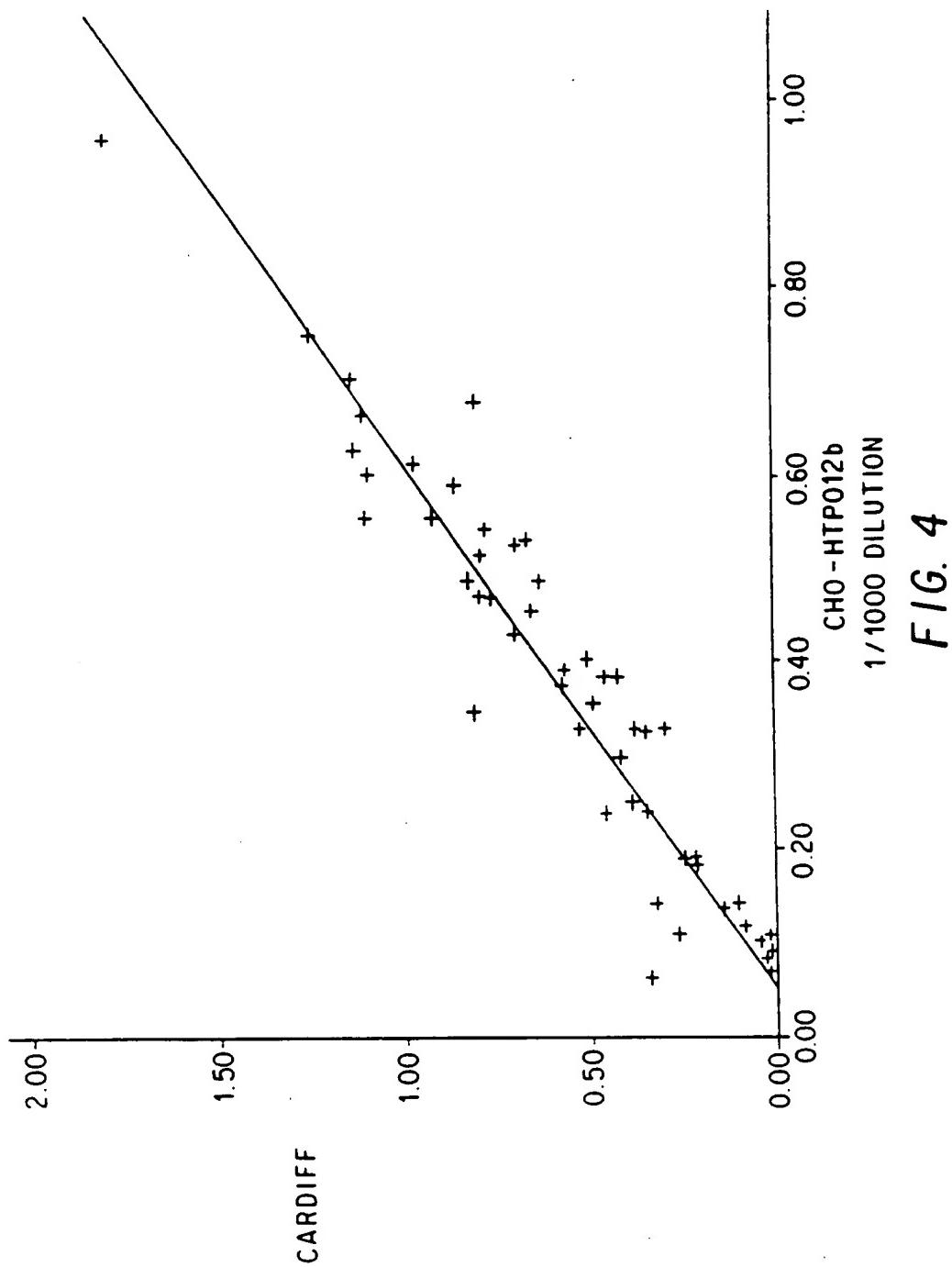


FIG. 4

SUBSTITUTE SHEET

7/27

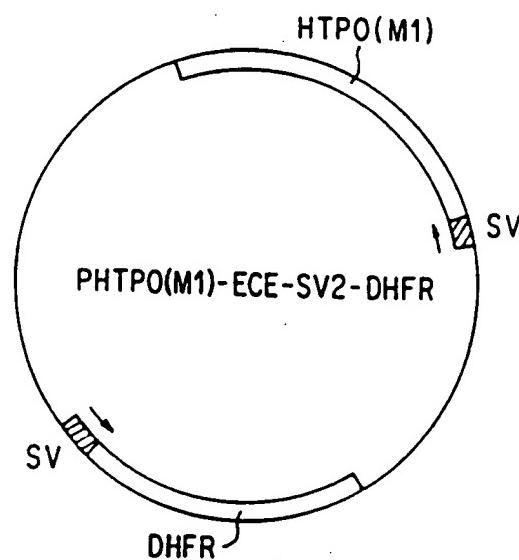


FIG. 8

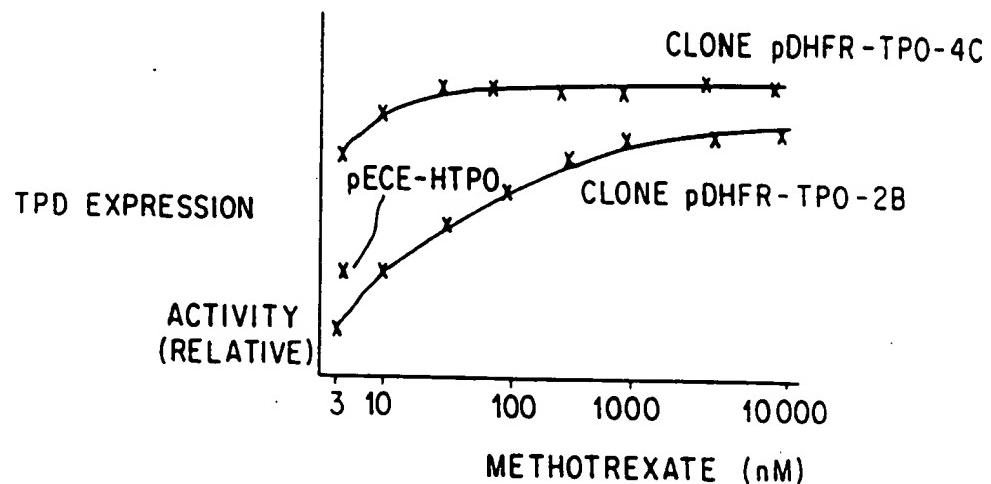


FIG. 5

SUBSTITUTE SHEET

8/27

2611

2662

AGG CTC CCC CGG GTC ACT TGG ATC UCC ATG TAG CCT GCT CIG CIG ATC G pHIPD-BS

Eco RI

Stop Stop

AGG CTC CCC CGG GTC ACT TGA ATT UCC ATG TAG CCT GCT CIG CIG ATC G pHIPD(MI)-BS

FIG. 6

SUBSTITUTE SHEET

9/27

GAG GCA ATT GAG GCG CCC ATT TCA	27	54 GAA GAG TTA CAG CCG TGA AAA TTA CTG AGC ... ...
AGT GCA GTT GGC TGA GAA GAG GAA AAA AGA ATG AGA GCG CTG GCT GTG CTG TCT	81	108 MET Arg Ala Leu Ala Val Leu Ser
GTC ACG CTG GTT ATG GCC TGC ACA GAA GCC TTC TTC CCC TTC ATC TCG AGA GGG	135	162 Val Thr Leu Val Met Ala Cys Thr Glu Ala Phe Phe Pro Phe Ile Ser Arg Gly
AAA GAA CTC CTT TGG GGA AAG CCT GAG GAG TCT CGT GTC TCT AGC GTC TTG GAG	189	216 Lys Glu Leu Leu Trp Gly Lys Pro Glu Glu Ser Arg Val Ser Ser Val Leu Glu
GAA AGC AAG CGC CTG GTG GAC ACC GCC ATG TAC GCC ACG ATG CAG AGA AAC CTC	243	270 Glu Ser Lys Arg Leu Val Asp Thr Ala Met Tyr Ala Thr Met Gln Arg Asn Leu
AAG AAA AGA GGA ATC CTT TCT GGA GCT CAG CTT CTG TCT TTT TCC AAA CTT CCT	297	324 Lys Lys Arg Gly Ile Leu Ser Gly Ala Gln Leu Leu Ser Phe Ser Lys Leu Pro
GAG CCA ACA AGC GGA GTG ATT GCC CGA GCA GCA GAG ATA ATG GAA ACA TCA ATA	351	378 Glu Pro Thr Ser Gly Val Ile Ala Arg Ala Ala Glu Ile Met Glu Thr Ser Ile
CAA GCG ATG AAA AGA AAA GTC AAC CTG AAA ACT CAA CAA TCA CAG CAT CCA ACG	405	432 Gln Ala Met Lys Arg Lys Val Asn Leu Lys Thr Gln Gln Ser Gln His Pro Thr
GAT GCT TTA TCA GAA GAT CTG CTG AGC ATC ATT GCA AAC ATG TCT GGA TGT CTC	459	486 Asp Ala Leu Ser Glu Asp Leu Leu Ser Ile Ile Ala Asn Met Ser Gly Cys Leu

FIG. 7A

SUBSTITUTE SHEET

10/27

513 CCT TAC ATG CTG CCC CCA AAA TGC CCA AAC ACT TGC CTG GCG AAC AAA TAC AGG  
 Pro Tyr Met Leu Pro Pro Lys Cys Pro Asn Thr Cys Leu Ala Asn Lys Tyr Arg 540

567 CCC ATC ACA GGA GCT TGC AAC AAC AGA GAC CAC CCC AGA TGG GGC GCC TCC AAC  
594 Pro Ile Thr Gly Ala Cys Asn Asn Arg Asp His Pro Arg Trp Gly Ala Ser Asn

621 648  
ACG GCC CTG GCA CGA TGG CTC CCT CCA GTC TAT GAG GAC GGC TTC AGT CAG CCC  
Thr Ala Leu Ala Arg Trp Leu Pro Pro Val Tyr Glu Asp Gly Phe Ser Gin Pro

675 CGA GGC TGG AAC CCC GGC TTC TTG TAC AAC GGG TTC CCA CTG CCC CCG GTC CGG  
702 Arg Gly Trp Asn Pro Gly Phe Leu Tyr Asn Gly Phe Pro Leu Pro Pro Val Arg

GAG GTG ACA AGA CAT GTC ATT CAA GTT TCA AAT GAG GTT GTC ACA GAT GAT GAC  
729 756  
Glu Val Thr Arg His Val Ile Gln Val Ser Asn Glu Val Val Thr Asp Asp Asp

783 810  
CGC TAT TCT GAC CTC CTG ATG GCA TGG GGA CAA TAC ATC GAC CAC GAC ATC GCG  
Arg Tyr Ser Asp Leu Leu MET Ala Trp Gly Gln Tyr Ile Asp His Asp Ile Ala

837 864

TTC ACA CCA CAG AGC ACC AGC AAA GCT GCC TTC GGG GGA GGG TCT GAC TGC CAG  
Phe Thr Pro Gln Ser Thr Ser Lys Ala Ala Phe Gly Gly Gly Ser Asp Cys Gln

891 918

ATG ACT TGT GAG AAC CAA AAC CCA TGT TTT CCC ATA CAA CTC CCG GAG GAG GCC  
 Met Thr Cys Glu Asn Gln Asn Pro Cys Phe Pro Ile Gln Leu Pro Glu Glu Ala

945 972

CGG CCG GCC GCG GGC ACC GCC TGT CTG CCC TTC TAC CGC TCT TCG GCC GCC TGC  
Arg Pro Ala Ala Gly Thr Ala Cys Leu Pro Phe Tyr Arg Ser Ser Ala Ala Cys

**FIG. 7B**

11/27

999 1026  
GGC ACC GGG GAC CAA GGC GCG CTC TTT GGG AAC CTG TCC ACG GCC AAC CCG AGG  
Gly Thr Gly Asp Gln Gly Ala Leu Phe Gly Asn Leu Ser Thr Ala Asn Pro Arg  
  
1053 1080  
CAG CAG ATG AAC GGG TTG ACC TCG TTC CTG GAC GCG TCC ACC GTG TAT GGC AGC  
Gln Gln Met Asn Gly Leu Thr Ser Phe Leu Asp Ala Ser Thr Val Tyr Gly Ser  
  
1107 1134  
TCC CCG GCC CTA GAG AGG CAG CTG CGG AAC TGG ACC AGT GCC GAA GGG CTG CTC  
Ser Pro Ala Leu Glu Arg Gln Leu Arg Asn Trp Thr Ser Ala Glu Gly Leu Leu  
  
1161 1188  
CGC GTC CAC GGC CGC CTC CGG GAC TCC GGC CGC GCC TAC CTG CCC TTC GTG CCG  
Arg Val His Gly Arg Leu Arg Asp Ser Gly Arg Ala Tyr Leu Pro Phe Val Pro  
  
1215 1242  
CCA CGC CGG CCT GCG GCC TGT GCG CCC GAG CCC GGC AAC CCC GGA GAG ACC CGC  
Pro Arg Ala Pro Ala Ala Cys Ala Pro Glu Pro Gly Asn Pro Gly Glu Thr Arg  
  
1269 1296  
GGG CCC TGC TTC CTG GCC GGA GAC GGC CGC GCC AGC GAG GTC CCC TCC CTG ACG  
Gly Pro Cys Phe Leu Ala Gly Asp Gly Arg Ala Ser Glu Val Pro Ser Leu Thr  
  
1323 1350  
GCA CTG CAC ACG CTG TGG CTG CGC GAG CAC AAC CGC CTG GCC GCG GCG CTC AAG  
Ala Leu His Thr Leu Trp Leu Arg Glu His Asn Arg Leu Ala Ala Leu Lys  
  
1377 1404  
GCC CTC AAT GCG CAC TGG AGC GCG GAC GCC GTG TAC CAG GAG GCG CGC AAG GTC  
Ala Leu Asn Ala His Trp Ser Ala Asp Ala Val Tyr Gln Glu Ala Arg Lys Val  
  
1431 1458  
GTG GGC GCT CTG CAC CAG ATC ATC ACC CTG AGG GAT TAC ATC CCC AGG ATC CTG  
Val Gly Ala Leu His Gln Ile Ile Thr Leu Arg Asp Tyr Ile Pro Arg Ile Leu

FIG. 7C

SUBSTITUTE SHEET

12/27

1485 1512  
GGA CCC GAG GCC TTC CAG CAG TAC GTG GGT CCC TAT GAA GGC TAT GAC TCC ACC  
Gly Pro Glu Ala Phe Gln Gln Tyr Val Gly Pro Tyr Glu Gly Tyr Asp Ser Thr

1539 1566  
GCC AAC CCC ACT GTG TCC AAC GTG TTC TCC ACA GCC GCC TTC CGC TTC GGC CAT  
Ala Asn Pro Thr Val Ser Asn Val Phe Ser Thr Ala Ala Phe Arg Phe Gly His  
\*

1593 1620  
GCC ACG ATC CAC CCG CTG GTG AGG AGG CTG GAC GCC AGC TTC CAG GAG CAC CCC  
Ala Thr Ile His Pro Leu Val Arg Arg Leu Asp Ala Ser Phe Gln Glu His Pro

1647 1674  
GAC CTG CCC GGG CTG TGG CTG CAC CAG GCT TTC TTC AGC CCA TGG ACA TTA CTC  
Asp Leu Pro Gly Leu Trp Leu His Gln Ala Phe Phe Ser Pro Trp Thr Leu Leu

1701 1728  
CGT GGA GGT GGT TTG GAC CCA CTA ATA CGA GGC CTT CTT GCA AGA CCA GCC AAA  
Arg Gly Gly Leu Asp Pro Leu Ile Arg Gly Leu Leu Ala Arg Pro Ala Lys

1755 1782  
CTG CAG GTG CAG GAT CAG CTG ATG AAC GAG GAG CTG ACG GAA AGG CTC TTT GTG  
Leu Gln Val Gln Asp Gln Leu Met Asn Glu Glu Leu Thr Glu Arg Leu Phe Val

1809 1836  
CTG TCC AAT TCC AGC ACC TTG GAT CTG GCG TCC ATC AAC CTG CAG AGG GGC CGG  
Leu Ser Asn Ser Ser Thr Leu Asp Leu Ala Ser Ile Asn Leu Gln Arg Gly Arg  
\*

1863 1890  
GAC CAC GGG CTG CCA GGT TAC AAT GAG TGG AGG GAG TTC TGC GGC CTG CCT CGC  
Asp His Gly Leu Pro Gly Tyr Asn Glu Trp Arg Glu Phe Cys Gly Leu Pro Arg

1917 1944  
CTG GAG ACC CCC GCT GAC CTG AGC ACA GCC ATC GCC AGC AGG AGC GIG GCC GAC  
Leu Glu Thr Pro Ala Asp Leu Ser Thr Ala Ile Ala Ser Arg Ser Val Ala Asp

## FIG. 7D

SUBSTITUTE SHEET

13/27

1971	1998
AAG ATC CTG GAC TTG TAC AAG CAT CCT GAC AAC ATC GAT GTC TGG CTG GGA GGC Lys Ile Leu Asp Leu Tyr Lys His Pro Asp Asn Ile Asp Val Trp Leu Gly Gly	
2025	2052
TTA GCT GAA AAC TTC CTC CCC AGG GCT CGG ACA GGG CCC CTG TTT GCC TGT CTC Leu Ala Glu Asn Phe Leu Pro Arg Ala Arg Thr Gly Pro Leu Phe Ala Cys Leu	
2079	2106
ATT GGG AAG CAG ATG AAG GCT CTG CGG GAC GGT GAC TGG TTT TGG TGG GAG AAC Ile Gly Lys Gln Met Lys Ala Leu Arg Asp Gly Asp Trp Phe Trp Trp Glu Asn	
2133	2160
AGC CAC GTC TTC ACG GAT GCA CAG AGG CGT GAG CTG GAG AAG CAC TCC CTG TCT Ser His Val Phe Thr Asp Ala Gln Arg Arg Glu Leu Glu Lys His Ser Leu Ser	
2187	2214
CGG GTC ATC TGT GAC AAC ACT GGC CTC ACC AGG GTG CCC ATG GAT GCC TTC CAA Arg Val Ile Cys Asp Asn Thr Gly Leu Thr Arg Val Pro Met Asp Ala Phe Gln	
2241	2268
GTC GGC AAA TTC CCC GAA GAC TTT GAG TCT TGT GAC AGC ATC ACT GGC ATG AAC Val Gly Lys Phe Pro Glu Asp Phe Glu Ser Cys Asp Ser Ile Thr Gly Met Asn	
2295	2322
CTG GAG GCC TGG AGG GAA ACC TTT CCT CAA GAC GAC AAG TGT GGC TTC CCA GAG Leu Glu Ala Trp Arg Glu Thr Phe Pro Gln Asp Asp Lys Cys Gly Phe Pro Glu	
2349	2376
AGC GTG GAG AAT GGG GAC TTT GTG CAC TGT GAG GAG TCT GGG AGG CGC GTG CTG Ser Val Glu Asn Gly Asp Phe Val His Cys Glu Glu Ser Gly Arg Arg Val Leu	
2403	2430
GTG TAT TCC TGC CGG CAC GGG TAT GAG CTC CAA GGC CGG GAG CAG CTC ACT TGC Val Tyr Ser Cys Arg His Gly Tyr Glu Leu Gln Gly Arg Glu Gln Leu Thr Cys	
2457	2484
ACC CAG GAA GGA TGG GAT TTC CAG CCT CCC CTC TGC AAA GAT GTG AAC GAG TGT Thr Gln Glu Gly Trp Asp Phe Gln Pro Pro Leu Cys Lys Asp Val Asn Glu Cys	

**FIG. 7E****SUBSTITUTE SHEET**

14/27

2511 2538  
GCA GAC GGT GCC CAC CCC CCC TGC CAC GCC TGT GCG AGG TGC AGA AAC ACC AAA  
Ala Asp Gly Ala His Pro Pro Cys His Ala Ser Ala Arg Cys Arg Asn Thr Lys

2565 2592  
GGC GGC TTC CAG TGT CTC TGC GCG GAC CCC TAC GAG TTA GGA GAC GAT GGG AGA  
Gly Gly Phe Gln Cys Leu Cys Ala Asp Pro Tyr Glu Leu Gly Asp Asp Gly Arg

2619 2646  
ACC TGC GTA GAC TCC GGG AGG CTC CCT CGG GTG ACT TGG ATC TCC ATG TCG CTG  
Thr Cys Val Asp Ser Gly Arg Leu Pro Arg Val Thr Trp Ile Ser Met Ser Leu

2673 2700  
GCT GCT CTG CTG ATC GGA GGC TTC GCA GGT CTC ACC TCG ACG GTG ATT TGC AGG  
Ala Ala Leu Leu Ile Gly Gly Phe Ala Gly Leu Thr Ser Thr Val Ile Cys Arg

2727 2754  
TGG ACA CGC ACT GGC ACT AAA TCC ACA CTG CCC ATC TCG GAG ACA GGC GGA GGA  
Trp Thr Arg Thr Gly Thr Lys Ser Thr Leu Pro Ile Ser Glu Thr Gly Gly

2781 2808  
ACT CCC GAG CTG AGA TGC GGA AAG CAC CAG GCC GTA GGG ACC TCA CCG CAG CGG  
Thr Pro Glu Leu Arg Cys Gly Lys His Gln Ala Val Gly Thr Ser Pro Gln Arg

2835 2862  
GCC GCA GCT CAG GAC TCG GAG CAG GAG AGT GCT GGG ATG GAA GGC CGG GAT ACT  
Ala Ala Ala Gln Asp Ser Glu Gln Glu Ser Ala Gly Met Glu Gly Arg Asp Thr

2889 2916  
CAC AGG CTG CCG AGA GCC CTC TGA GGG CAA AGT GGC AGG ACA CTG CAG AAC AGC  
His Arg Leu Pro Arg Ala Leu ^^^

2943 2970  
TTC ATG TTC CCA AAA TCA CCG TAC GAC TCT TTT CCA AAC ACA GGC AAA TCG GAA

2997 3024  
ATC AGC AGG ACG ACT GTT TTC CCA ACA CGG GTA AAT CTA GTA CCA TGT CGT AGT

3051  
TAC TCT CAG GCA TGG ATG AAT AAA TGT TAT AGC TGC AAA AAA AAA AAA  
^ ^ ^ ^

FIG. 7F

SUBSTITUTE SHEET

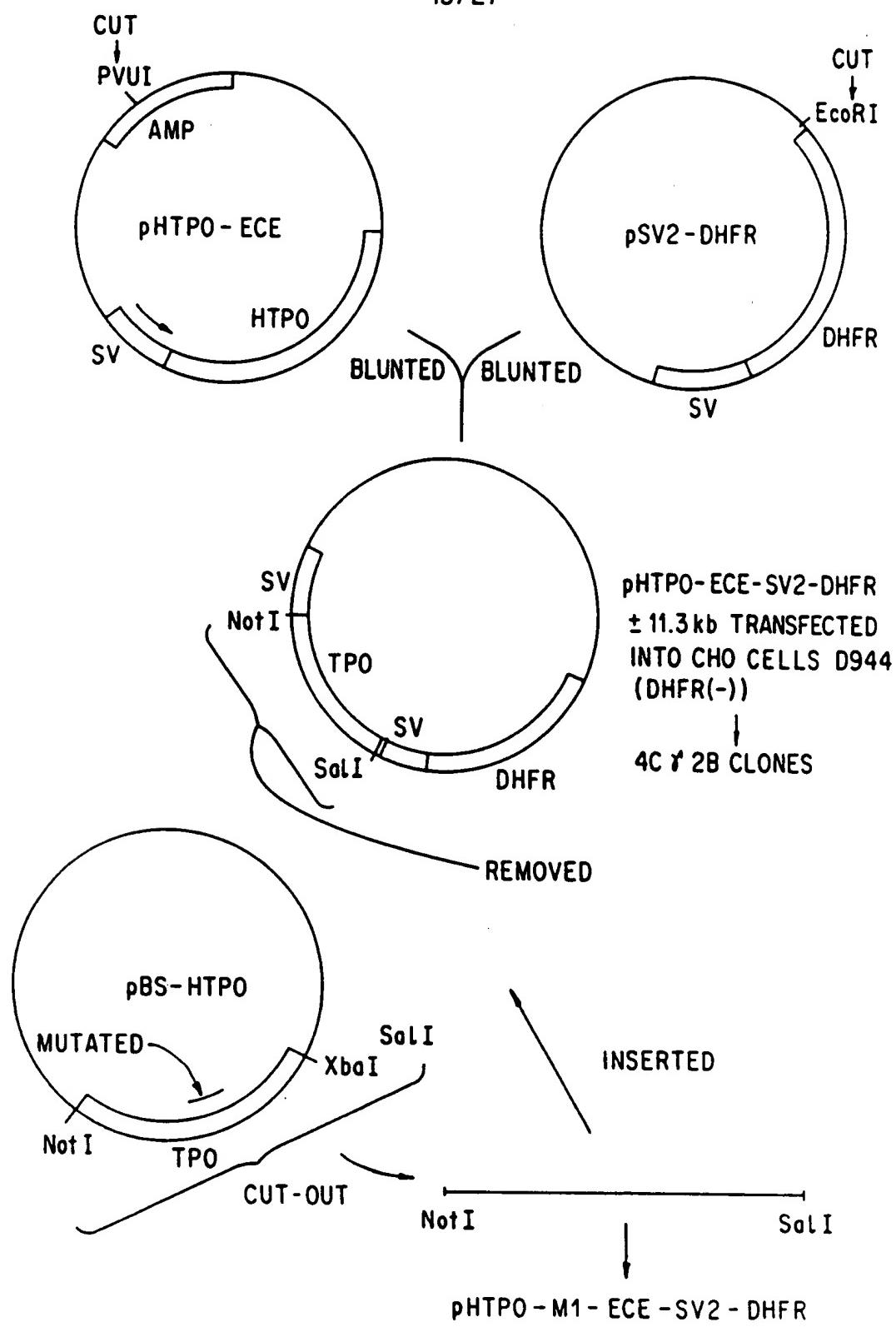


FIG. 9  
SUBSTITUTE SHEET

16/27

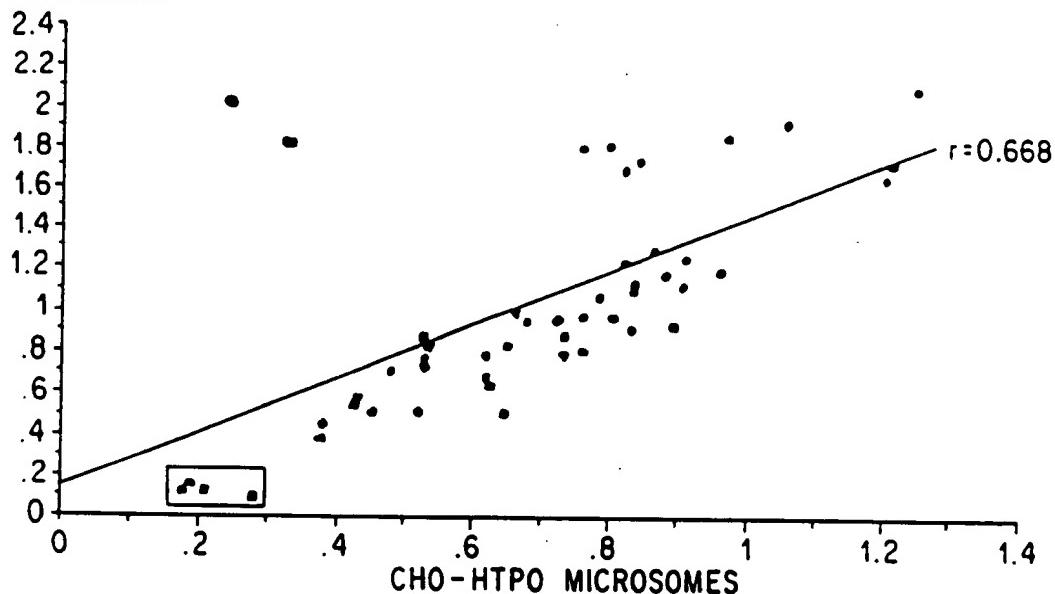
HUMAN THYROID  
MICROSOMES

FIG. 10A

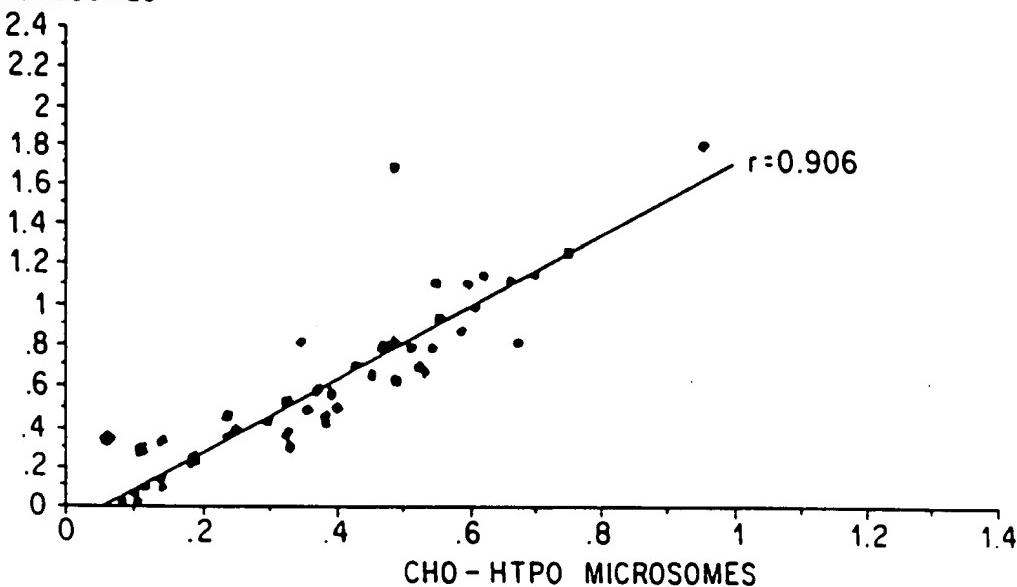
HUMAN THYROID  
MICROSOMES

FIG. 10B

SUBSTITUTE SHEET

17/27

HUMAN THYROID  
MICROSOMES

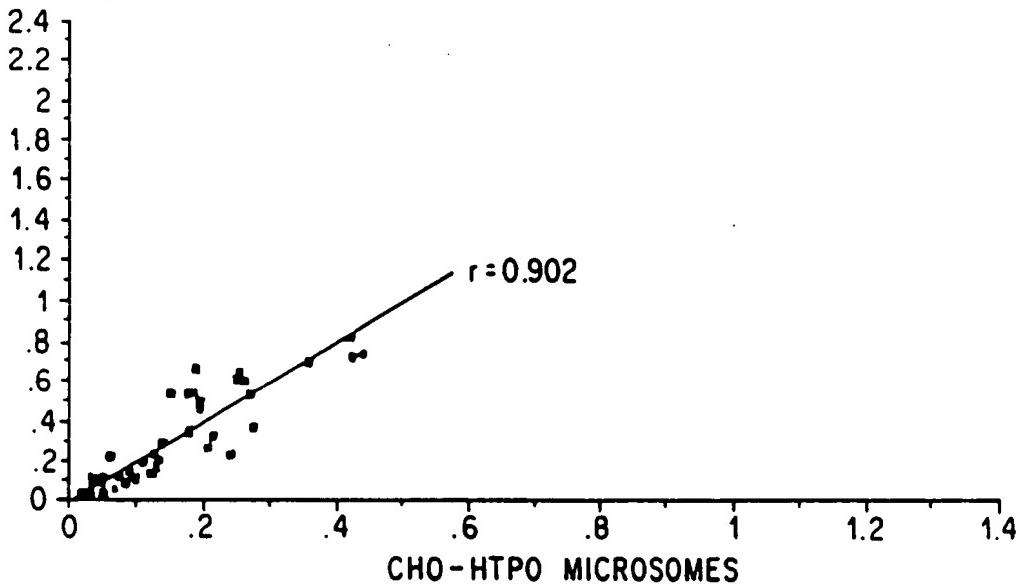
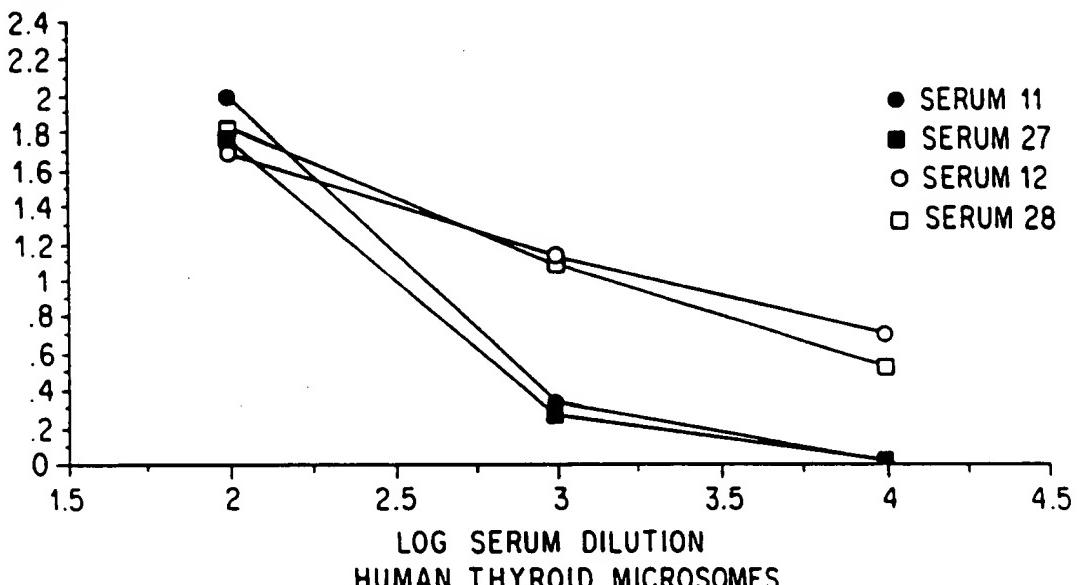


FIG. 10C

OPTICAL  
DENSITY



LOG SERUM DILUTION HUMAN THYROID MICROSONES

FIG. 11A

SUBSTITUTE SHEET

18/27

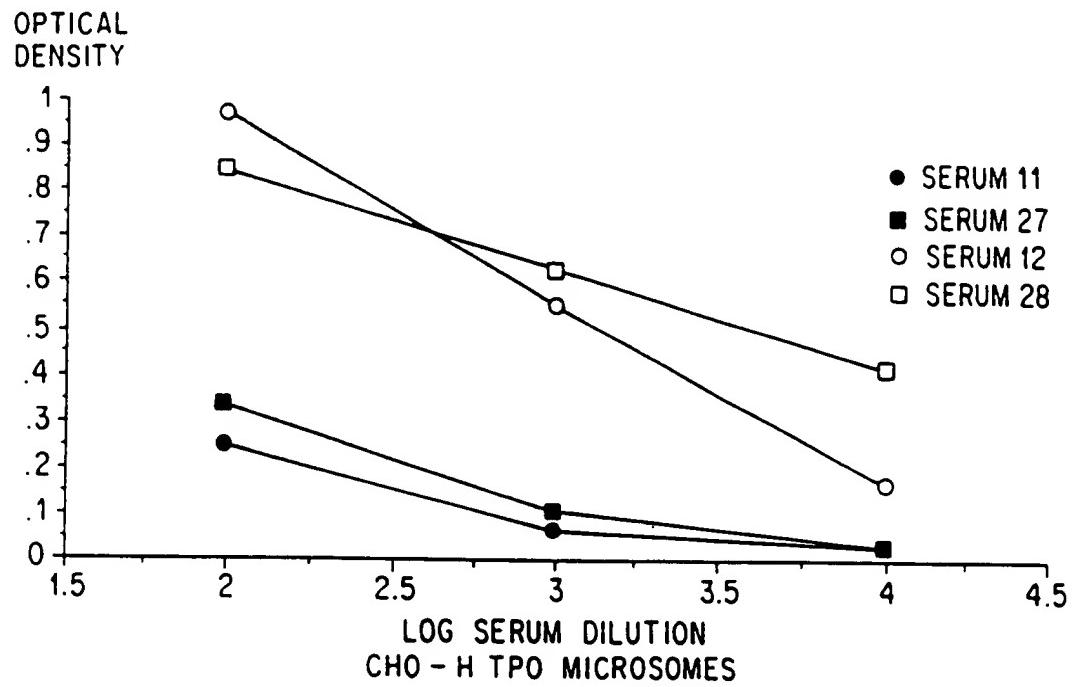
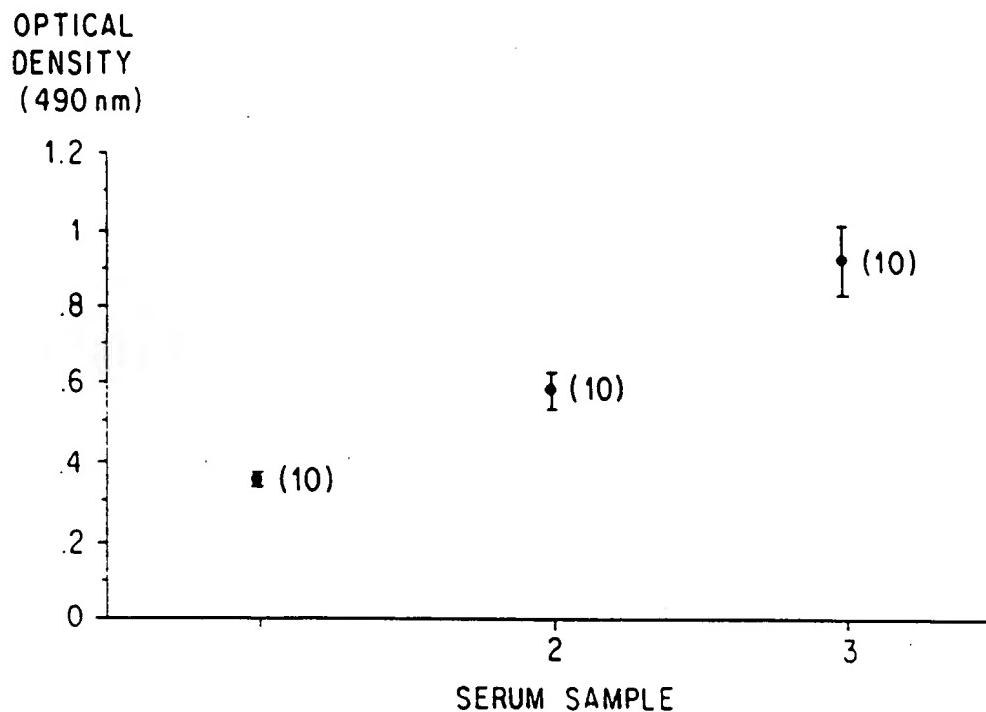


FIG. 11B

FIG 12  
SUBSTITUTE SHEET

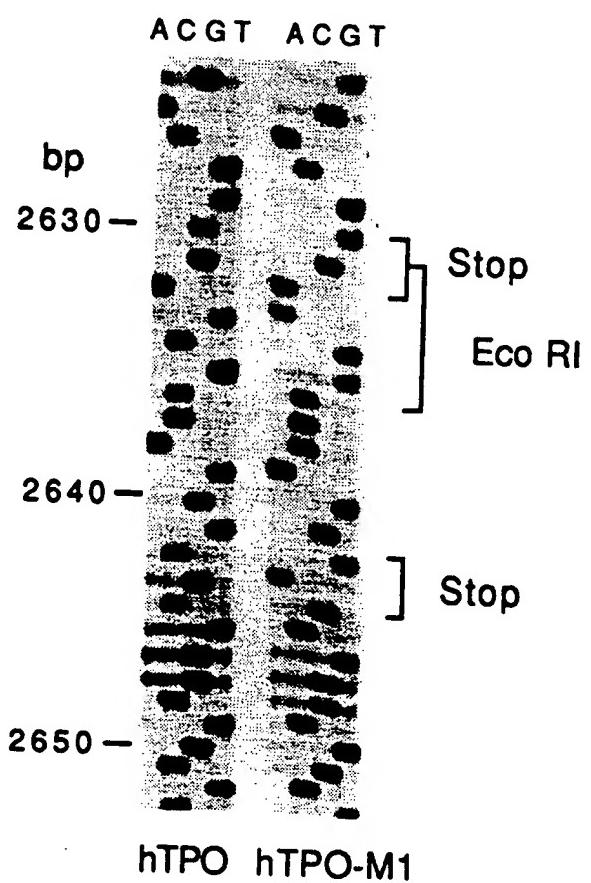


FIG. 13

SUBSTITUTE SHEET

20/27

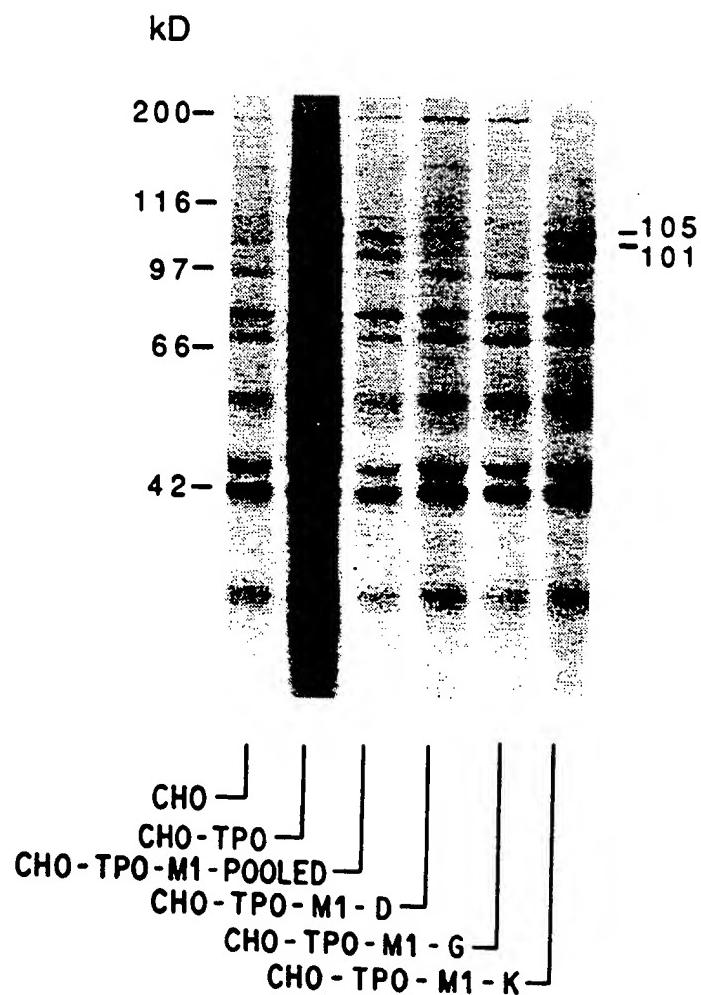


FIG. 14A

SUBSTITUTE SHEET

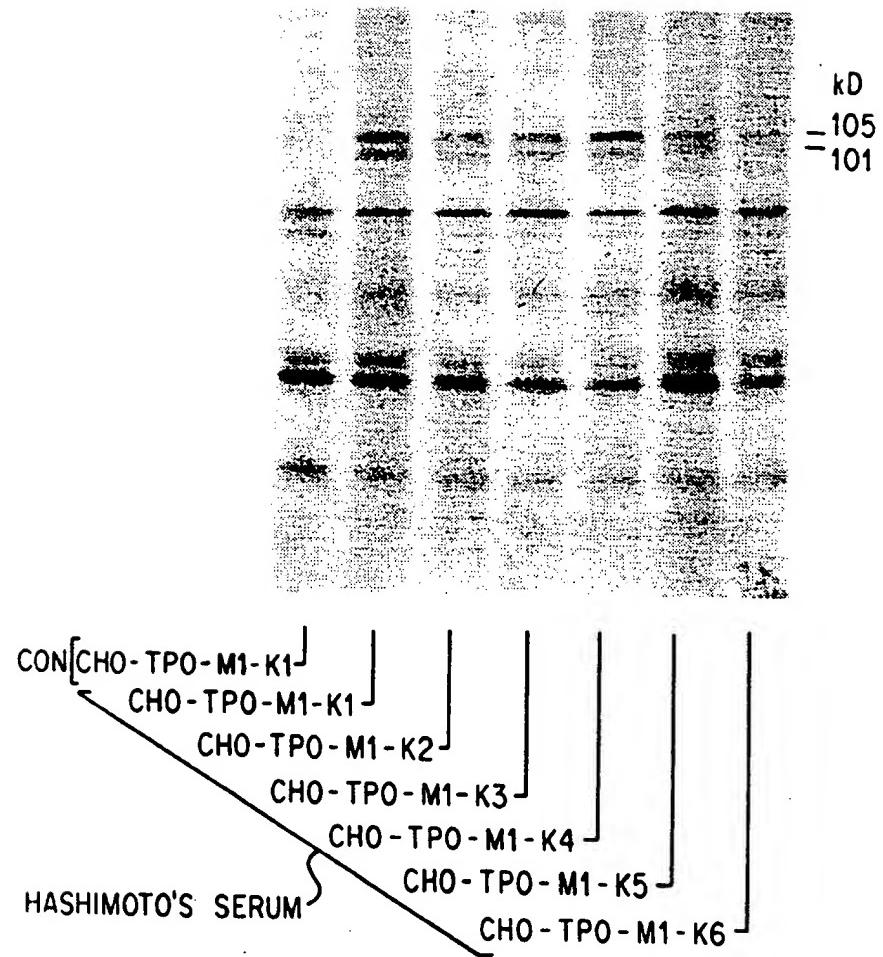


FIG. 14B

SUBSTITUTE SHEET

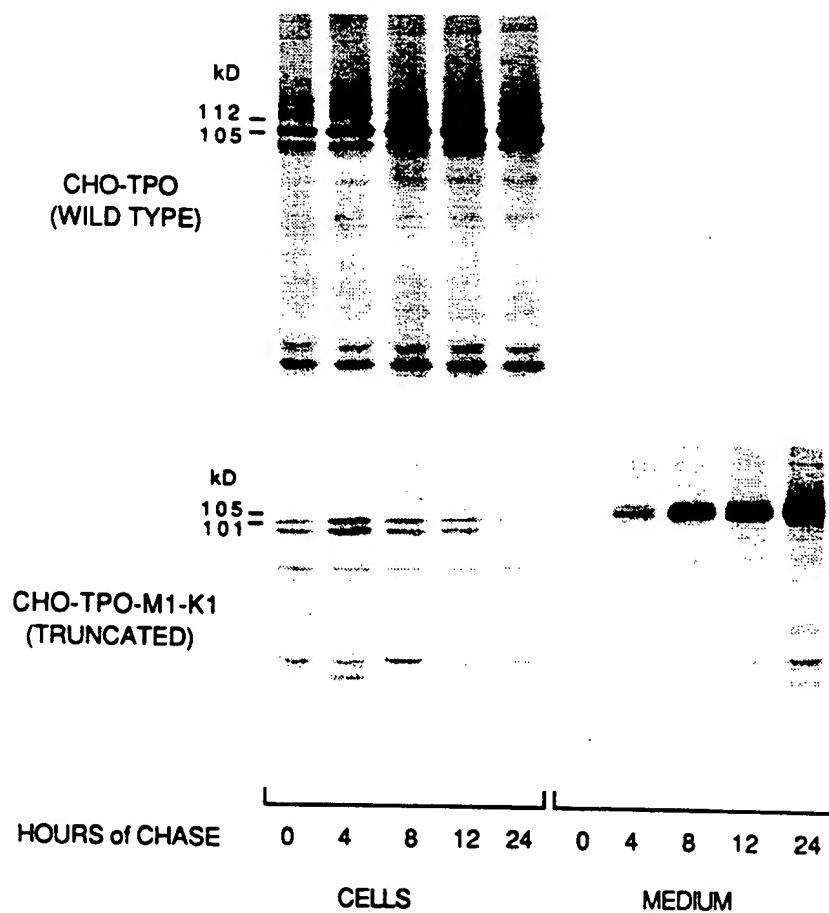


FIG. 15

## SUBSTITUTE SHEET

23/27

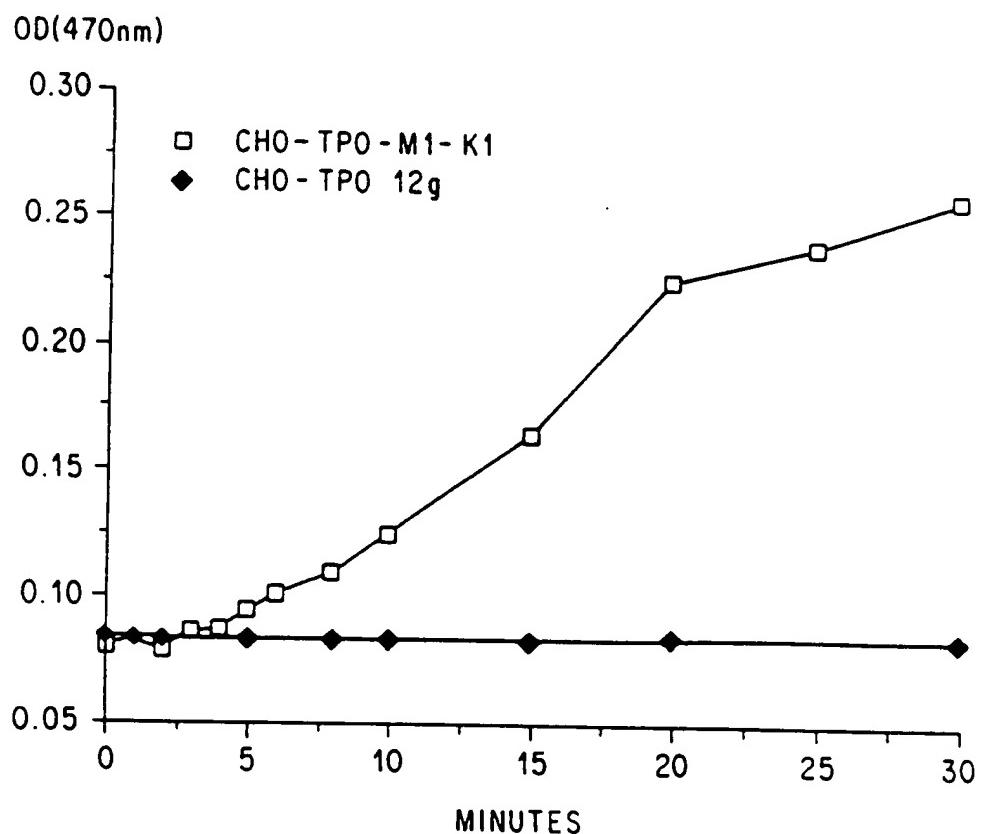


FIG. 16

SUBSTITUTE SHEET

24/27

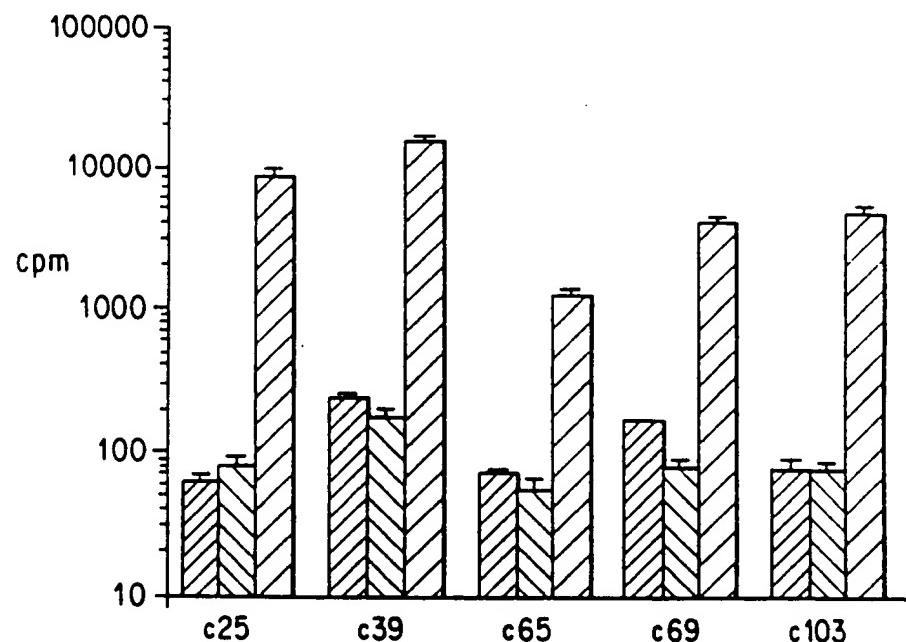


FIG. 17A

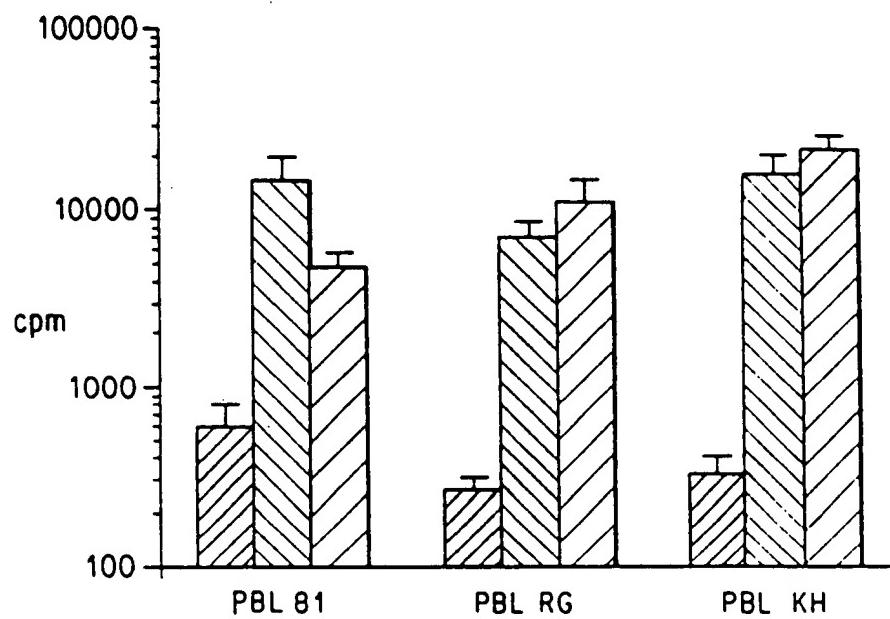


FIG. 17B

SUBSTITUTE SHEET

25/27

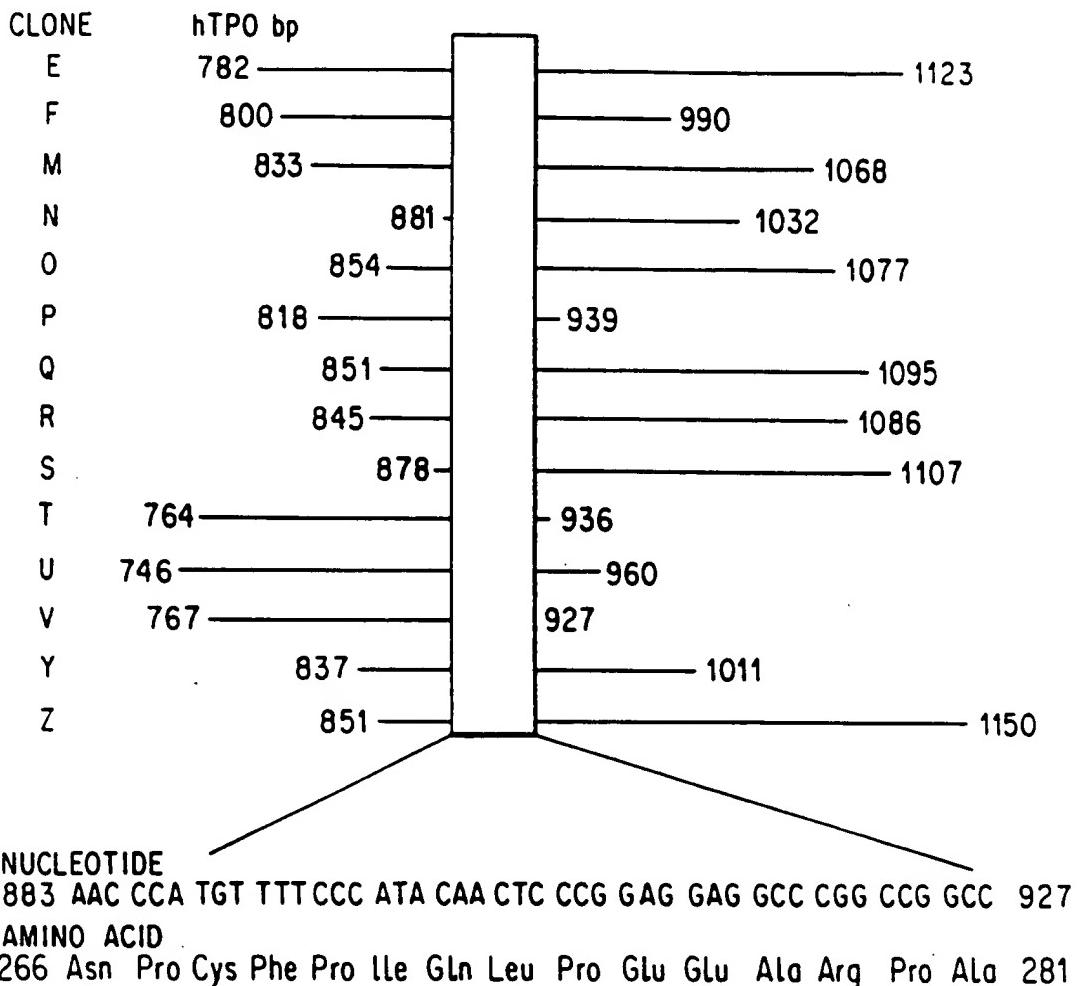
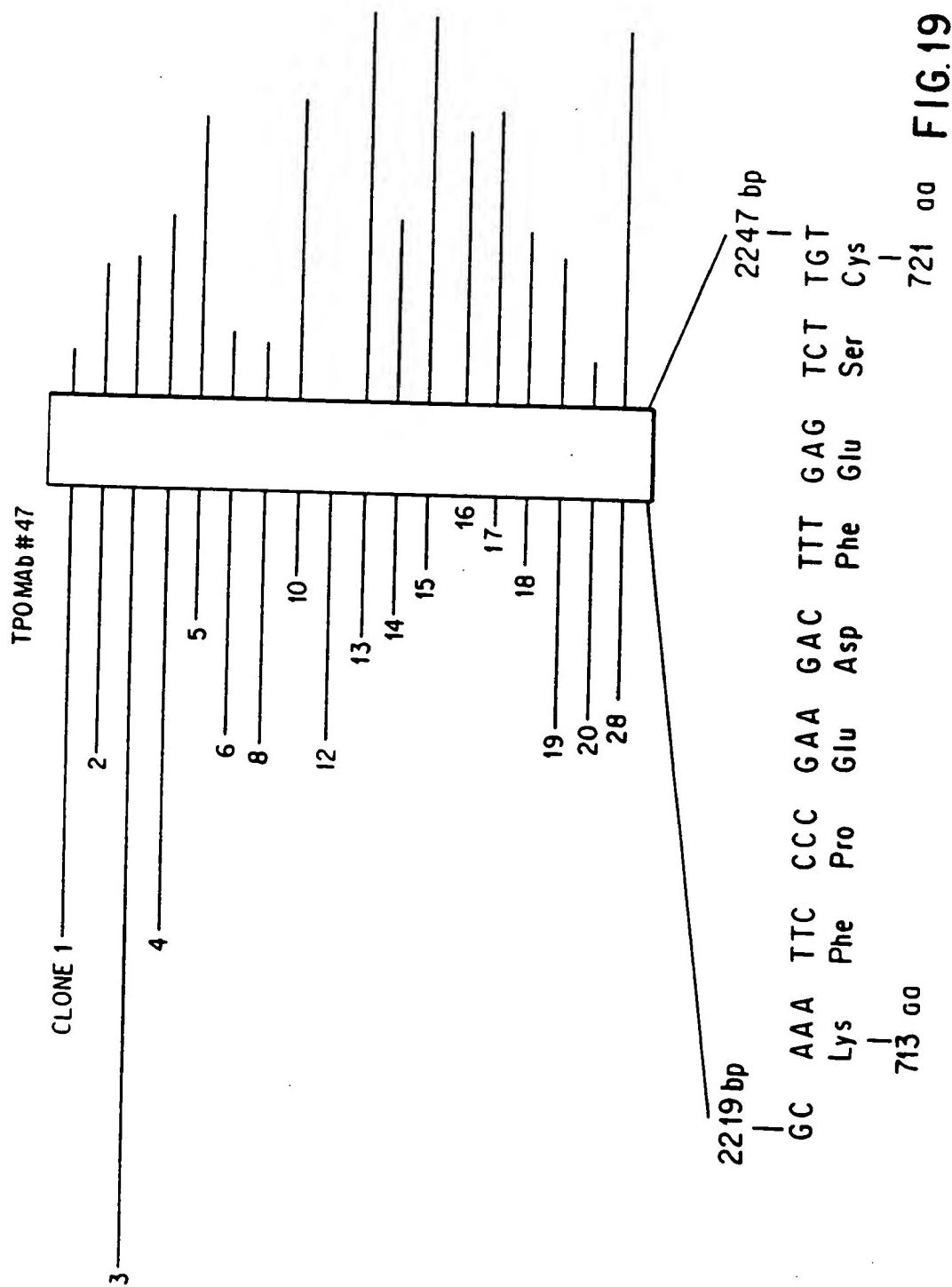


FIG. 18

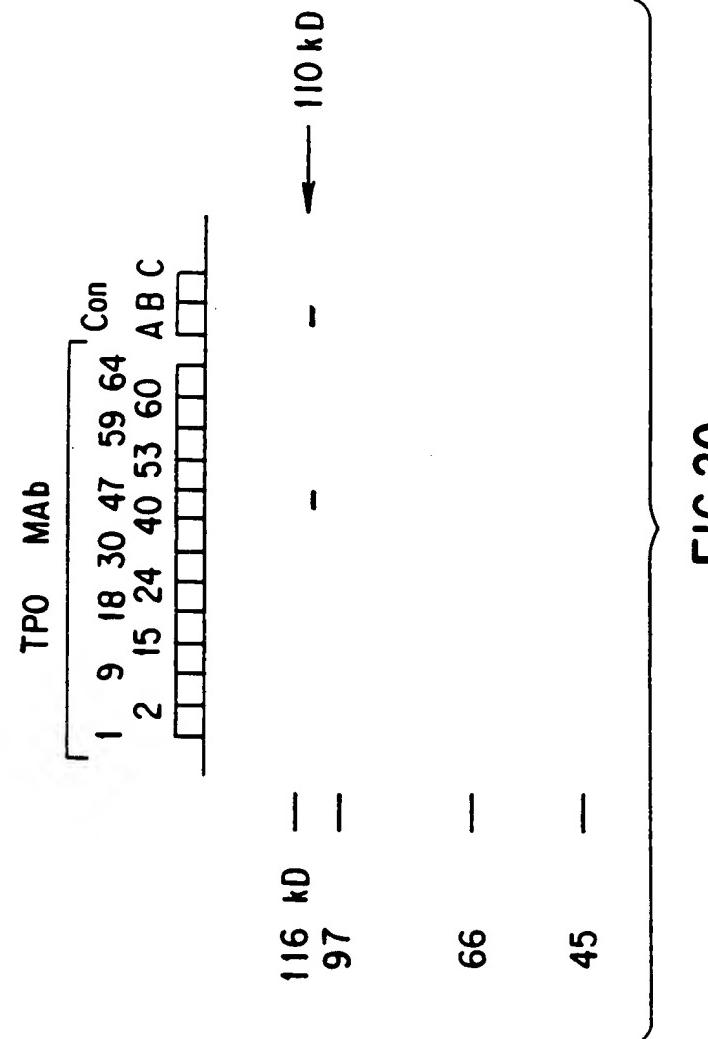
SUBSTITUTE SHEET

26/27



SUBSTITUTE SHEET

27/27



SUBSTITUTE SHEET